MAST CELL LIPID DROPLETS AS A STORAGE SITE OF ARACHIDONIC ACID FOR EICOSANOID BIOSYNTHESIS

Pro gradu

Annika Tentke

Helsingin yliopisto

Biotieteiden laitos

Fysiologian ja neurotieteen osasto

2014

Abstract

Human mast cells are potent effector cells in host defense mechanisms of innate and acquired immunity, including inflammatory diseases such as asthma and atherosclerosis. Mast cells originate from pluripotent hematopoietic progenitors in the bone marrow. Activation of mast cells by different stimuli triggers the release of a large range of mediators, including de novo synthesized eicosanoids which are highly biologically active lipid mediators. Eicosanoids derive from the omega-6 polyunsaturated fatty acid, arachidonic acid (AA) which is usually esterified at the sn-2 position of membrane phospholipids. Human mast cells actually contain a significant amount, if not even the majority, of arachidonic acid incorporated in triglycerides (TGs) which are stored in their cytoplasmic lipid droplets. While the liberation of arachidonic acid from phospholipids by phospholipase family members is well-studied, the hydrolysis of AA-containing triglycerides for the use of eicosanoid biosynthesis has not been studied until very recently.

The aim of this study is the elucidation of molecular mechanisms involved in the generation of eicosanoids in human mast cells with particular emphasis on lipid droplets as a source and/or site of lipid mediator biogenesis. The major prostanoid released by activated mast cells is the eicosanoid prostaglandin D2 (PGD_2). The putative enzymes involved in arachidonic acid liberation from triglycerides included (hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). We were also interested about hematopoietic prostaglandin D synthase (HPGDS), the key enzyme in the production of D and J series of prostanoids. The methods used included cell culture work and siRNA transfections; basic molecular biological methods such as RNA isolation, cDNA synthesis and qPCR; basic molecular biochemical methods including immunoblotting and ELISA; as well as immunofluorescence staining.

The increase in the amount of PGD_2 released from mast cells sensitized with human IgE (1µg/ml) and activated by polyclonal rabbit anti-human IgE (1µg/ml) was immediate and most prominent after one hour of activation, and slowly decreased to basal levels at 48h post-activation. siRNA transfection affected the amount of enzyme DNA in mast cells and the amount of PGD_2 released. HSL, ATGL and HSL+ATGL double knockdown all reduced the amount of PGD_2 released in acute (5 to 30 minutes) term activation compared to control cells. However, no significant changes were observed in the mRNA expression levels of ATGL, HSL, CGI-58, HPGDS or COX-1 under mast cell activation. The only significant changes in mRNA expression levels were observed with COX-2. The relative expression of HPGDS increased in IgE treated mast cells compared to control

treated cells and the expression was even greater in mast cells treated also with αIgE. Both ATGL and HPGDS were recognized throughout the cytosolic area in the non-activated Ctrl cells. Although HPGDS located also in the circumference of mast cells, no clear localization of HPGDS was observed in the circumference of mast cell lipid droplets as expected based on previous findings about the localizations within the lipid droplets of the enzymes involved in the synthesis of leukotrienes and prostaglandins.[1]

Tiivistelmä

Syöttösolut saavat alkunsa hematopoieettisista CD34 -pinta-antigeenille positiivisista esiasteista. Syöttösolut ovat osa kehon synnynnäistä ja hankittua immuunipuolustusjärjestelmää ja ne tunnetaan parhaiten klassisten allergiaoireiden aiheuttajina. Syöttösolut osallistuvat kuitenkin myös sepelvaltimotaudin, astman ja aineenvaihduntasairauksien kehittymiseen. Syöttösolujen aktivaatio johtaa erilaisten solu- ja lipidivälittäjäaineiden kuten eikosanoidien synteesiin (de novo) ja vapautumiseen. Eikosanoidit ovat biologisesti aktiivisia kudoshormoneita ja lipidivälittäjäaineita, joiden tiedetään muodostuvan kun monityydyttymätön omega-6 rasvahappo, arakidonihappo hydrolysoidaan solukalvorakenteiden fosfolipideistä, yleensä asemasta 2 (Sn2). Arakidonihapon vapautumista fosfolipideistä on tutkittu paljon ja vapautumisreitit ovat hyvin tunnettuja. Sen sijaan eikosanoidien biosynteesin mekanismia syöttösolujen lipidipartikkeleiden triglyserideistä ei ole tutkittu aiemmin. Ihmisten syöttösolujen sytosolissa sijaitsevien lipidipartikkeleiden ytimien triglyserideissä tiedetään kuitenkin olevan runsaasti arakidonihappoa.

Tämän tutkielman tarkoitus on perehtyä ihmisen syöttösolujen eikosanoidien biosynteesin molekyylitason mekanismeihin. Erityisesti mielenkiinnon kohteena ovat syöttösolut lipidivälittäjäaineiden muodostuspaikkana. Arakidonihapon vapautumisessa triglyserideistä ovat olennaisessa osassa hormoni-sensitiivinen lipaasi (HSL) ja rasvakudoksen triglyseridilipaasi (ATGL). Syöttösolujen pääasiallisesti vapauttama eikosanoidi on prostaglandiini D2 (PGD_2). Hematopoieettinen prostaglandiini D syntaasi (HPGDS) on tärkeä tämän D- sarjan prostaglandiinin entsymaattisessa muodostuksessa. Tutkimuksessa käytettiin soluviljelytöitä ja siRNA transfektiota syöttösolujen entsyymigeenien hiljentämiseen; RNA:n eristystä, cDNA synteesiä, qPCR:ää entsyymiproteiinien sekvenssin monistamiseksi; immunodetektiota entsyymiproteiinien ja ELISAa muodostuvan PGD_2 määrän kvantitointiin; ja immunofluoresenssivärjäyksiä lipidipartikkeliden osoittamiseen ja entsyymiproteiinien sijainnin määrittämiseen syöttösoluissa.

Syöttösolujen herkistäminen IgE:llä (1µg/ml) ja aktivointi polyklonaalisella αIgE:llä (1µg/ml) sai aikaan vapautuvan PGD_2:n määrän nopean ja huomattavan suurenemisen. Vapautuvan PGD_2:n määrän kasvu oli voimakkaimmillaan tunnin kuluttua aktivaatiosta ja palasi lähtötasolle 48 tuntia aktivaation jälkeen. siRNA-transfektio vaikutti entsyymi DNA:n ja vapautuvan PGD_2:n määrään. HSL, ATGL ja HSL+ATGL hiljennykset alensivat vapautuvan PGD_2 määrää kontrollisoluihin verrattuna akuutin (5-30 minuutin) aktivaation jälkeen. mRNA-tasolla ei havaittu merkittäviä muutoksia ATGL, HSL, CGI-58, HPGDS tai COX-1 ekspressiossa aktivaation jälkeen. Ainoa

merkittävä muutos havaittiin COX-2- entsyymin mRNA-ekspressiossa. HPGDS:n suhteellinen ekspressio lisääntyi IgE:llä käsitellyissä syöttösoluissa kontrolleihin verrattuna, ja oli tätäkin voimakkaampaa myös αIgE:llä käsitellyissä soluissa. Immunofluoresenssivärjäyksissä vasta-aineet tunnistivat sekä ATGL että HPGDS ei-aktivoitujen kontrollisolujen sytosolissa. Vaikka HPGDS tunnistettiin myös solujen ulkoreunoilla, entsyymi ei sijoittunut erityisesti syöttösolujen lipidipartikkeleiden ympärille, vaikka näin oli syytä odottaa aiempien tutkimusten perusteella.[1]

Key words:

Mast cell • Lipid droplet • Triglyceride • Arachidonic acid • Eicosanoids • Hormone-sensitive lipase • Hematopoietic prostaglandin D synthase • Prostaglandin D2

*Abbreviations**

A

AA = arachidonic acid

ACSL = long chain acyl-CoA synthetase

ATGL = adipose triglyceride lipase

B

BSA = bovine serum albumin

C

CGI-58 = a comparative gene identification-58 or α/β-Hydrolase Domain -Containing Protein 5, also known as 1-acylglycerol-3-phosphate O-acyltransferase ABHD5

cAMP = cyclic adenosine monophosphate, cyclic AMP or 3'-5'-cyclic adenosine monophosphate

COX = cyclooxygenase 1 and 2 also known as prostaglandin H synthase or PGHS 1 and 2

cPLA2 = cytosolic phospholipase A2

D

DAG = diacylglycerol

E

EDTA = ethylenediaminetetraacetic acid

ELISA = enzyme-linked immunosorbent assay

G

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

H

HPGDS = hematopoietic prostaglandin D synthase a.k.a terminal hematopoietic prostaglandin D2 synthase

HSL = hormone-sensitive lipase

K

KIT = kit-ligand, KITL or KL also known as stem cell factor (SCF), a cytokine that binds to the c-

Kit receptor (CD117)

L

LD = lipid droplet a.k.a lipid body

LPGDS = lipocalin-type prostaglandin D synthase

LSDP5 = lipid storage droplet protein, also known as lipid droplet scaffold protein

5-LO = arachidonate 5-lipoxygenase, 5-lipoxygenase

15-LO (15-1) = 15-lipoxygenase-1

M

MC = mast cell

MITF = microphthalmia-associated transcriptor factor, a basic-helix-loop-helix leucine zipper transcription factor

N

NEFA = non-esterified fatty acid

P

PGD_2 = Prostaglandin D2

PGH = Prostaglandin H

PLIN1 = Perilipin, also known as lipid droplet-associated protein

PLIN2 = perilipin 2, also known as Adipose differentiation-related protein, ADFP or adipophilin

PKA = Protein kinase A

Q

qPCR = quantitative polymerase chain reaction

R

RasGRP4 = RAS guanyl releasing protein 4

RT-PCR = reverse transcriptase polymerase chain reaction

*The abbreviations used were selected because they are well-known or commonly used in scientific articles of related topics and on the other hand were as unambiguous as possible having no other meaning (in biology).

Table of Contents

1 Introduction

1.1 Mast cells

Human mast cells are present in almost all vascularized tissues including skin, mucosal tissue of lungs and digestive tract, arterial intima, mouth, nose and conjunctiva. They are most common at the sites exposed to the external environment. Mast cells (Fig. 1) are oval cells of approximately 10 µm in size with densely packed and metachromatically staining granules. The number of mast cells in healthy individuals is quite stable, but pathologic conditions can disturb this homeostasis leading to mastocytosis i.e. the accumulation of mast cells and release of mast cell mediators.[2] Disorders of mast cell proliferation are known, but the ability to study mast cells has been limited because mature mast cells can only be extracted from tissue sites –from lungs, skin, uterus and intestine[3] and because of a lack of means to maintain human mast cells in long term cultures.[4]

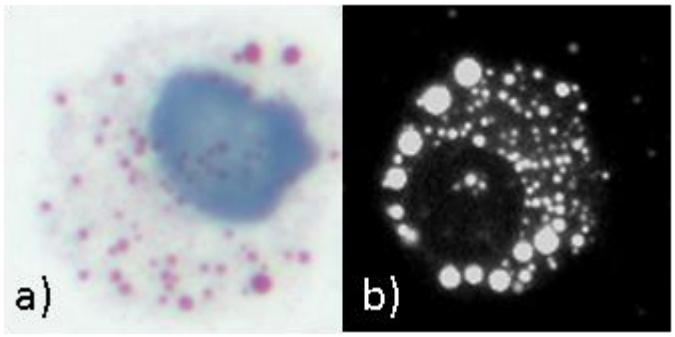


Figure 1: Human mast cells on their 5th week of culture. The round nucleus stained blue is surrounded by lipid droplets of various sizes. (a) Neutral lipid droplets were visualized by ORO stainings or (b) by the fluorescent dye BODIPY493/503 ® in mast cells where lipid droplet formation was enhanced by exogenous treatment with AA:BSA -complexes (250 µM for 18 hours at +37°C).

1.2 Origin, migration, development and differentiation of mast cells

There are two mast cell phenotypes, the mucosal and the connective tissue type. The connective tissue type produces both tryptase and chymase (TC-MC), whereas mucosal mast cells (T-MC) express tryptase only. All mast cells originate from pluripotent hematopoietic progenitors in the bone marrow, circulate as mast cell progenitors (MCPs) and acquire their final maturity and develop their granules in the target tissues, where they ultimately reside. Mast cell survival and maturation is guided by KITL, a ligand for c-Kit receptor expressed on the mast cell surface.[5] KITL is a chemokine expressed by stromal mesenchymal cells[6,7] and the only cytokine and adherence factor absolutely vital for the development of mature mast cells.[8] Cell membrane bound KITL or its soluble isoforms are positively chemotactic for mast cells and their progenitors.[2] Besides being a chemotactic factor, KITL also elicits cell to cell, and cell to substratum adhesion, facilitates cell

proliferation, differentiation and maturation.[2] Binding of KITL to c- Kit receptor induces the homodimerization of two receptor monomers, thus facilitating their activation.[2,9] Signalling downstream from c-Kit induces well-known pathways including phospholipases.[9] Additional mast cell maturation and survival factors are interleukin 3 (IL-3), Th2-derived IL-4 and IL-9, and various other factors.[6,7]

1.3 Mast cell mediators

Mast cells are documented to play a major role in both innate and acquired immunity including inflammatory diseases such as allergy, intestinal disease and atherosclerosis as well as lifestyle or metabolic diseases.[10] Mast cells are able to rapidly and selectively produce appropriate mediators for physiological and innate immune responses, they can enhance effector cell recruitment because of their location near blood vessels and they are able to modify immune responses against infectious agents in an antibody dependent manner.[3] Mast cells can be activated by non-immunological stimuli (e.g. pollen, bacteria, virus, parasites, chemicals, radiation) or by immunological stimuli, including specific antigens or molecules, complement proteins or by other transmembrane stimuli.[11] Three main classes of mediators are produced in mast cells: preformed granule-associated mediators such as vasoactive amine histamine and serine proteases; newly generated lipid mediators produced independently from above mentioned granules; and cytokines, chemokines and other growth factors.[1,3,12-14] Mast cells express a large variety of cell surface receptors which enable a specific response to different stimuli via the release of these biologically active mediators.

The best-studied signalling pathways of mast cells are those mediated either through high-affinity IgE receptor FcϵRI (Fcε receptor type I) which plays a classical role in acute hypersensitivity reactions, including asthma and other allergic disorders or those activated when calcium inonophores are added to the cells.[5] IgE usually exists as a monomer. In the classical IgE/FcεRI pathway the binding of an antigen to IgE which is already tightly bound to FcεRI on mast cell surface, causes the pairing of two IgE-molecules. This pairing or crosslinking leads to aggregation of the FcεRIs, a rapid exocytosis of cytoplasmic secretory granules via a process termed 'degranulation' and to concomitant release of mediators such as PGD_2[15] and generation of diacylglycerol (DAG),[8] the substrate of hormone sensitive lipase (HSL). DAG generation has been associated to morphological changes taking place during mast cell activation.[8] The cytoplasmic secretory organelles contain histamine and neutral serine proteases such as tryptase and chymase typical for mast cells,[1,16] and they are responsible for the type I allergy a.k.a hypersensitivity or

classical allergy, although a ”T-helper cell 2 -type of response” and B-cell activation and switch to IgE isotype is also needed. In this condition the immune response targets against harmless or useful substances.

Activation via another receptor called c-Kit also known as CD117 leads to formation of the de-novo synthesized eicosanoids including PGD_2, thromboxane, platelet-activating factor, leukotriene (LT) B4 and leukotriene C4 (LTC4) which are all highly biologically active lipid mediators.[11] Knowledge of the mechanisms for their secretion is incomplete, but all major mast cell lipid mediators are known to derive enzymatically from a common precursor, arachidonic acid (AA), an ω-6 polyunsaturated fatty acid with 20 carbon atoms and double bonds at positions 5,8,11 and 14 (5,8,11,14-all-cis-Eicosatetraenoic acid) for which reason they are called ‘eicosanoids’. The arachidonic acid can be obtained either from the diet or it can be enzymatically derived from linoleic acid 18:2(n-6).[17] Newly formed eicosanoids are lipid soluble and can move out from the cell and affect neighboring cells, diffuse to the extracellular space, be re-incorporated to new phospholipids or degraded enzymatically.[18] Eicosanoids function as tissue hormones, signalling lipids and lipid mediators of inflammation,[19] messenger molecules that are released from the parent cell in response to tissue injury and act at nanomolar concentrations in an autocrine and paracrine fashion.[14] Some of them help to stimulate the action of cells involved in the immune response while others help to "turn off" an immune response when it is no longer needed. The ultimate reason for the inflammation is to protect the tissue from further damage and to clean it from damaged or dead cells and the cell signalling molecules produced by them in order to end the inflammation and heal the tissue.

1.4 Newly generated mast cell lipid mediators include Prostaglandin D_2 (PGD_2)

Prostanoids are a subclass of eicosanoids consisting of prostaglandins, thromboxanes and prostacyclins. They are mediators of inflammatory and anaphylactic reactions, and they have important roles in maintaining cardiovascular homeostasis and protecting the cardiac tissue against oxidative injury in hypoxic conditions.[14] Prostaglandins are a group of enzymatically derived lipid compounds composed of 20 carbon atoms and a 5-carbon ring.[20] PGD_2 is the major eicosanoid produced in central nervous system (CNS) and peripheral tissues[21] and its functions include promotion of sleep, thermoregulation, olfactory functions, hormone release, nociception (“pain sense”) in the CNS, prevention of platelet aggregation and induction of vasodilation and bronchoconstriction.[5] In peripheral tissues PGD_2 is mainly produced by activated mast cells,[13] but

in some extend also by leukocytes, T helper type 2 (Th2) cells and dendritic cells.[21] Prostaglandins E_2 and I_2 are said to be the pro-inflammatory prostanoids that enhance vasodilation, vascular permeability and oedema formation[14] while PGD_2 has both inflammatory and anti-inflammatory capacity.[21] The effect of PGD_2 depends on the combination of disease etiology, cell types included and the expression profile of eicosanoid receptors in the recipient cells.[21,22] PGD_2 has a short half-life[5] and it is readily dehydrated both in vitro and in vivo[5] or further metabolized to 9α, 11β-PGF_2.[11] PGD_2 can also be transported to the extracellular space by a PG transporter.[11]

1.5 PGD_2 biosynthesis

1.5.1 COX-1 and COX-2 produce Prostaglandin $PG(H)_2$ from arachidonic acid

Several enzymes use arachidonic acid as their substrate for eicosanoid biosynthesis and free arachidonic acid has three main metabolic pathways to form eicosanoids.[14] These pathways include enzymes called cyclooxygenase (COX), lipoxygenases (LOX) and cytochrome P450 enzymes.[14] The COX –pathway (Fig. 2) and the concomitant production of prostaglandins is the focus of this study while the two other pathways are not discussed. COXs are known to metabolize arachidonic acid released by phospholipase A2 to prostaglandin $PG(H)_2$.[21-23] COXs are bifunctional enzymes that have both bis-dioxygenase and peroxidase nature[14] and they are the rate limiting enzymes in the production of PGD_2 from arachidonic acid.[20] COXs catalyze cyclooxygenase (bis-oxygenase) reactions in which arachidonic acid and two molecular oxygens combine to form PGG_2, the physiologically important substrate of cyclooxygenase and hydroperoxidase reaction where PGG_2 is reduced to PGH_2 with the help of two electrons.[24]

There are two main isoforms of COXs: COX-1 and COX-2.[14] Although both isoforms catalyze the same reaction and their gross kinetic properties (Km, Vmax) are similar, there are differences in the structure of the active site, sequence of the lipid binding domain and biology of the two isoforms.[24] In many cell types, COX-1 is localized to ER and nuclear membrane, while the COX-2 isoforms is even more concentrated in the nuclear membrane.[24] COX-1 is constitutively expressed in most cells and it is the source of prostanoids that serve to promote housekeeping functions vital for normal physiological activities of cells.[14] COX-2 is most of the time undetectable but can be rapidly induced by inflammatory stimuli such as pro-inflammatory cytokines, hormones and growth factors.[14,21] COX-2 messenger RNA levels are also known to rapidly degrade[20] after inflammatory stimuli.

All that said both COX-1 and -2 may contribute to the acute inflammatory response. The type of the isoform mainly responsible for the inflammation and the type of eicosanoids produced from the arachidonic acid depends first of all on the proteome within the cell and especially on the relative amounts of the two isoforms present in the tissue in question as well as on their downstream enzymes.[14,21,22] The synthetic profile can be further altered by means of inducible forms of certain synthases that require environmental or exogenous signals such as IL-8 for their activation.[14] Also the physical compartmentalization of the COX-1 and -2 enzymes with other enzymes next in the pathway might link the activity of a certain isoform with the synthesis of certain eicosanoids.[14] Major proportion of eicosanoids generated by mast cells consist of PGD_2 synthesized via COX pathway and leukotriene C4 (LTC_4) synthesized via 5-lipoxygenase pathway.[14,21]

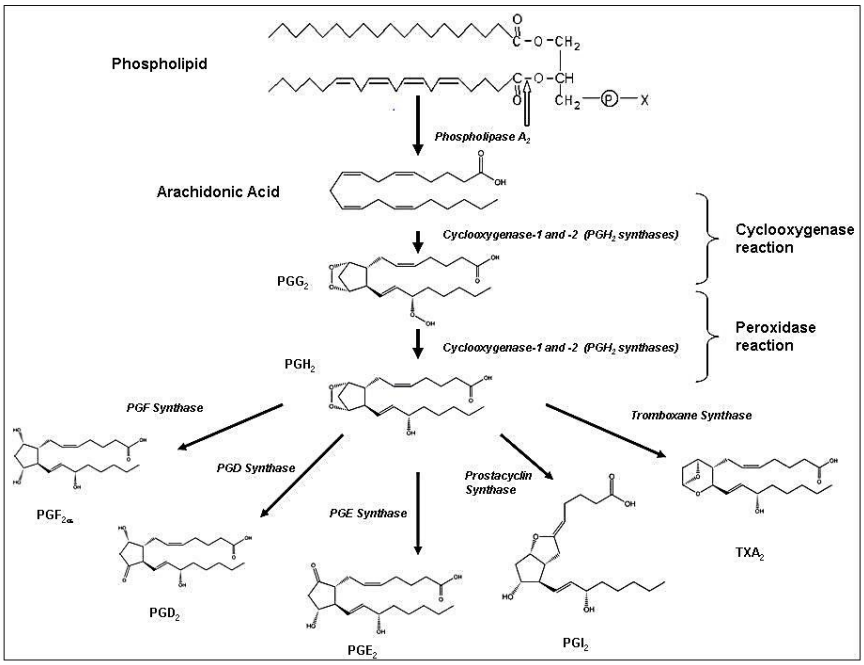


Figure 2: COX pathway leading to the production of prostaglandins. The two COX enzymes, COX-1 and COX-2, metabolize phospholipase A2-derived arachidonic acid to prostaglandin $PG(H)_2$, the common precursor for eicosanoids and substrate for other downstream enzymes. (http://www.intechopen.com/books/an-update-on-glomerulopathies-etiology-and-pathogenesis/glomerulonephritis-and-cellular-regulation-of-prostaglandin-synthesis 14.10.2013)

1.5.2 PGD synthases catalyze the formation of PGD_2 from prostaglandin $PG(H)_2$

1.5.2.1 LPGDS

Two distinct types of the PGD synthase (PGDS) have been identified; the lipocalin-type LPGDS and the hematopoietic enzyme HPGDS. Despite the differences in catalytic properties, amino acid sequence, gene and tertiary structure, cellular localization and tissue distribution, both enzymes isomerize PGH_2 (Fig. 2), the product of COX-enzymes and a common precursor for all prostanoids, to PGD_2 and its anti-inflammatory metabolite 15-deoxy- Δ12-14 PGJ_2 (15d-PGJ_2) in a specific, glutathione-dependent manner.[5,22,25] Lipocalin-type PGD synthase also known as glutathione-independent PGDS, β-trace protein, prostaglandin-H2 D-isomerase, the brain type enzyme or glutathione (GSH)-independent enzyme is a multifunctional protein,[25] that belongs to the lipocalin protein family that consists of lipophilic ligand carrier proteins and retinoic acid binding proteins.[5] LPGDS is localized in the central nervous system and male genital organs of various mammals and also in the human heart. In addition to its PGD_2-producing activity LPGDS functions as an extracellular transporter for small lipophilic molecules like for example haem metabolites, retinoids, thyroid hormones, steroids, flavonoids and saturated fatty acids, as an endogenous somnogen or an agent that promotes sleep, in allergic responses and in clearing the body quickly from harmful or otherwise unwanted lipophilic molecules.[26] LPGDS binding to haemin, biliverdin and bilirubin likely protect these from enzymatic catalysis.[26]

1.5.2.2 HPGDS

The hematopoietic enzyme HPGDS also known as the spleen type enzyme or GSH-requiring enzyme is a key enzyme in the production of D and J series of prostanoid. HPGDS has previously been identified to be expressed in antigen presenting cells, megakaryocytes, type 2 helper T lymphocytes, microglia and dendritic cells of many tissues.[5,20] HPGDS is localized in the mast cell cytosol[21] and it is approximately of the size of 26 kDa. Hyo et al. [27] have found that HPGDS is involved in activation and differentiation of mast cells. HPGDS can be up regulated[5] and induced by KIT in immature mouse bone marrow derived mast cells and the induction is further enhanced by IL-3, IL-9 and IL-10.[28] These mast cells also show highly increased PGD_2 production when cultured with KIT and IL-3, IL-9 or IL-10, sensitized with IgE and activated with αIgE.[28] The increase is dose-dependent for KIT and the interleukins[28]. IL-9 together with KIT enhances the long term viability of mast cells while IL-3 can inhibit the final stages of differentiation and maturation

of mast cells.[2] The regulatory mechanisms and the function of HPGDS in mast cells are still largely unknown.

1.6 Lipid droplets

Lipid droplets (Fig. 1) also known as lipid bodies, consist of a neutral lipid core surrounded by a monolayer of amphipathic lipids such as phospholipids and unesterified cholesterol and by proteins involved in the turnover of lipids as well as formation and trafficking of the lipid droplets.[19,29] The major phospholipids include lyso-phosphatidylcholine, phosphatidylcholine and sphingomyelin.[29] The proteins are either embedded in the membrane or associate with it through electrostatic interactions.[19] The lipid monolayer is fluid and allows lateral movement of the associated proteins.[30] Depending on the cell type, the core is composed mainly of triglycerides, cholesterol esters, other esters and diacylglycerol.[31,32] The neutral lipids in the core and the phospholipids in the surrounding monolayer supply fatty acids for various needs, and these fatty acids need to be replenished rapidly after usage by re-esterification or re-acylation reactions. Fatty acids are believed to cycle between the core and the surrounding lipid monolayer in order to maintain the homeostasis between triglycerides and phospholipids.[12] Metabolic functions of lipid droplets include anabolic reactions such as fatty acid synthesis and activation, biosynthesis of sterols and triglyceride molecules, and catabolic reactions such as hydrolysis, mobilization and modification of lipid droplet components.

Lipid droplets are found in almost every cell type and virtually all cell types are capable of accumulating and storing neutral lipids in lipid droplets the number of which increases in cells at sites of inflammation.[23,29,33] As with the cell membranes; the composition of lipid droplet macromolecules varies depending on the cell type and the metabolic state of the cell.[29] In adipocytes, lipid droplets store metabolic energy in the form of triglycerides, and hydrolysis of these triglycerides (TGs), known as lipolysis, provides energy during conditions such as fasting or starvation.[34] Excessive lipolysis can lead into accumulation of toxic lipid intermediates and oxidized lipids. This is why the cellular hydrolysis of triglycerides is carefully controlled to meet the prevailing needs.[34] Characterization of lipid droplets in some cell types has shown that they are active and important sites for arachidonic acid metabolism,[23,35] and a source of substrates for the eicosanoid biosynthesis.[1,19] Arachidonic acid-derived products are not stored, but instead are very unstable and synthesized de-novo when needed. In leukocytes a major proportion of arachidonic acid is found in lipid droplet triglycerides. For most other cell types the function and the biochemical content of lipid droplets is less well known[23] although disturbances in lipid droplet

metabolism are known to be involved in some of the most widespread human diseases, such as atherosclerosis and diabetes.[31,36]

1.7 Mast cell lipid droplets

According to current scientific articles lipid droplets in mast cells are evidently equivalent to those in other types of inflammatory cells.[29] However the cytoplasmic lipid droplets of mast cells are less well studied.[1] Mast cell lipid droplets can be recognized as electron-dense organelles of different sizes and increase in number during the differentiation of mast cells.[29] They are present in mast cells of healthy tissues as well as in mast cells which have been found at sites of inflammation.[23] The accumulation of lipid droplets in mast cells is a process that seems to be different from that of adipocytes.[1] Typically there are less than 10 lipid droplets per mast cell[1], but immunological challenge,[1] developmental abnormalities or environmental challenges can also lead to a rapid increase in the number of cytoplasmic lipid droplets in mast cells.[31] The developmental differentiation of CD34+ progenitor cells into mature mast cells is associated with an increase in number and size of their cytoplasmic lipid droplets.[29] Cytoplasmic lipid droplets are not co-secreted with the granules upon mast cell activation, but instead are granule- independent organelles.[29] Electron microscopic, auto radiographic studies of mast cells have demonstrated that exogenous radiolabeled arachidonic acid is incorporated into lipid droplet triglycerides and that the lipid droplet formation itself is also stimulated by unsaturated fatty acids, such as oleic acid and arachidonic acid.[29,33]

1.8 Mast cell lipid droplets could release arachidonic acid from two different sites

Arachidonic acid is known to control cell activation, metabolism, cell migration, proliferation and death by the induction of apoptosis.[33] The availability of free arachidonic acid is a rate-limiting step in the generation of mammalian eicosanoids.[37] In the resting cells arachidonic acid is esterified into glycerol and stored in the glycerophospholipids of the cell membrane and other membrane structures. The cellular concentrations of free arachidonic acid are low under physiological conditions.[14] Arachidonic acid can be released from different sub-cellular compartments such as the nuclear envelope, the phagosome, ER or lipid droplet membrane.[11,35] Free arachidonic can also be incorporated back to other (phospho)lipids or it can leave the cell by diffusion. Arachidonyl-coenzyme A (CoA) -synthase acts on free arachidonic acid and Coenzyme A to synthesize an

unsaturated fattyacyl-CoA regulating the eicosanoid production. The enzymatic products arachidonyl-CoA and HETE-CoA do not accumulate in the cells but are instead re-incorporated into (phospho) lipids.[38]

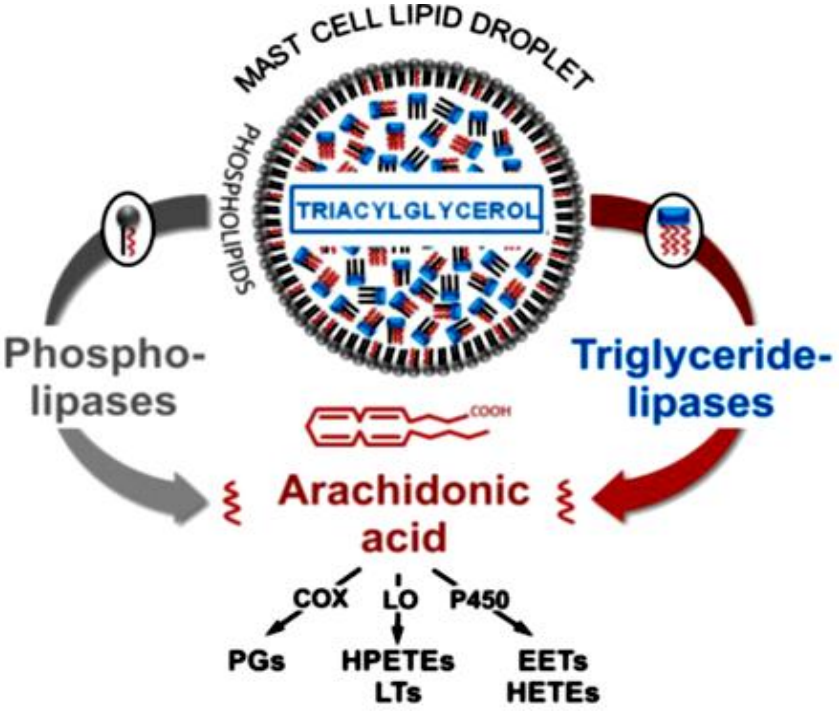


Figure 3: Mast cell arachidonic acid release. Both phospholipases and triglyceride (i.e. triacylglycerol) lipases can release arachidonic acid from mast cell lipid droplets (Dichlberger, A., Kovanen, P.T., Schneider, W.J.). [12]

1.8.1 Phospholipases release arachidonic acid from the phospholipid monolayer

In mast cells PGD_2 can be produced through three main steps from two different sites (Fig. 3). Free arachidonic acid released by phospholipases is oxidized by cyclooxygenases COX-1 and/or COX-2 to produce prostaglandins via PGH2; by lipoxygenases to produce hydroperoxyeicosatetraenoic acids (HPETEs) and leukotrienes; or by cytochrome P450 epoxygenase and ω-hydroxylase to produce epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), respectively. [12,19]

The action of different phospholipase enzymes, mainly activated cPLA2α[14,20,37] also known as group IVA PLA2,[33] release arachidonic acid from the sn-2 position of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) in membranous structures in response to a variety of immunoinflammatory stimuli.[37] Depending on cell type and stimulation conditions also other phospholipase A2 enzymes may contribute.[37] A rise in intracellular calcium level causes the resting state cPLA2-α to translocate to the vicinity of its arachidonic acid containing phospholipid substrates and the downstream enzymes of the arachidonic acid cascade[35] and allows the targeting and binding of cPLA2-α to membrane structures through its Ca^{2+} binding domain.[35,37] The Ca^{2+} -dependent[37] activation of cPLA2- α can be initiated by receptor mediated agonists, microorganisms, phagocytic particles and non-specific stimuli such as cell or tissue damage.[14] In addition to intracellular calcium flux also specific downstream signalling events such as phosphorylation by mitogen activated protein kinases (MAPKs) and ERK are needed to functionally activate the enzyme.[11] Lipid droplet-translocated and activated cPLA2-α becomes enzymatically active[35] and specifically recognizes the sn-2 acyl bond of amphipathic lipids in the lipid monolayer and catalytically hydrolyzes the bond releasing fatty acids (arachidonic acid) and lysophospholipids,[33] rapidly increasing the amount of free arachidonic acid. A correlation has been found to exist between the expression level of cPLA2α and the amount of PGD_2 released.[37] In epithelial cells stimulation with arachidonic acid or oleic acid has been shown to trigger the translocation of cPLA2-α as well.[35]

1.8.2 Arachidonic acid in triglycerides of mast cell lipid droplets

In addition to membrane phospholipids, the human mast cells contain a huge amount of arachidonic acid incorporated in triglycerides stored in the cores of their cytoplasmic lipid droplets.[29] Whereas the liberation of arachidonic acid from phospholipids by phospholipase family members is well-

studied, the hydrolysis of arachidonic acid containing triglycerides for the use of eicosanoid biosynthesis is less well understood. It is known that exogenous arachidonic acid can be incorporated into mast cell triglycerides, and that such incorporation enhances lipid droplet formation in the cells.[29] Thus, mast cell lipid droplets may serve as a cytoplasmic storage site for arachidonic acid.[29] Putative cytosolic lipases that release arachidonic acid from the triglycerides in the lipid droplet core include ATGL (adipose triglyceride lipase) and HSL (hormone sensitive lipase).

1.8.2.1 ATGL

Triglyceride lipases are enzymes capable of hydrolyzing ester linkages of triglyceride fatty acids. ATGL catalyzes the rate limiting reaction of triglyceride hydrolysis,[39] and initiates the lipolysis acting on triglycerides, releasing diacylglycerols and non-esterified free fatty acids (NEFAs), the latter of which in case of adipose cells are liberated into the blood circulation to provide energy for energy-poor tissues.[39] There is no report in the scientific literature so far that would state, that ATGL releases arachidonic acid from mast cell triglycerides in lipid droplets. ATGL belongs to a human patatin-like phospholipase gene family (PNPLAs) containing five members in vertebrates[19], and it is widely expressed in a variety of cells.[34] In adipocytes ATGL appears to be distributed between the cytoplasm and lipid droplets.[19] Induction of triglyceride hydrolysis requires binding of ATGL to the lipid droplet surface and its activation.[34]

1.8.2.2 HSL

The triglyceride lipase activity of ATGL is followed by the action of HSL on diacylglycerols.[19,40] HSL is an intracellular neutral lipase that exists as a functional dimer composed of homologous subunits. The dimer has greater enzymatic activity compared with monomeric HSL.[41] HSL is a multifunctional enzyme that can act on triglycerides,[40] diacylglycerols, monoacylglycerols, cholesterylesters and other water-soluble substrates. However, it shows preference for diacylglycerols.[41] Although HSL functions in hydrolysis of fatty acids in di- and triglycerides of lipid droplets, the enzyme preferentially releases polyunsaturated fatty acids, and has the capacity to hydrolyze diacylglycerols much faster than triglycerides (triacylglycerols).[40]

1.8.3 PAT proteins regulate the activity of HSL and ATGL

Lipolysis and lipid droplet metabolism is regulated by the composition of the lipid droplet-associated proteins on the lipid droplet surface. The most prominent proteins are PAT proteins.[29,34] The PAT protein family includes PLIN 1 also known as perilipin 1, perilipin 2 (PLIN 2) also known as adipophilin, perilipin 3 (PLIN 3) also known as TIP47, perilipin 4 (PLIN 4) also known as adipocyte Protein S3-12 and perilipin 5 (PLIN 5) also known MLDP (OXPAT/PAT-1)[19] and lipid storage droplet protein 5 also known as LSDP5. Regulation of PAT expression and hence also the activity of lipolytic enzymes is tissue specific.[34] Mammalian lipid droplets contain one or more of these six PAT proteins,[19] and PAT genes are transcribed also in human mast cells.[29] They regulate the basal and induced lipolysis of stored neutral lipids[19] by controlling the access of lipases to the lipid droplet core and the exposure of the triglycerides of lipid droplets to enzymatic actions of lipases[40] by means of lateral movement within the lipid droplet membrane[30] and on the other hand by serving as structural components that stabilize lipid droplets.[30] PAT proteins are also thought to serve as scaffolds via which recruitment and reorganization of other proteins to the lipid droplet surface occurs.[29] In non-activated cells (Fig. 4) perilipin coats the lipid droplet surface and interacts with CGI-58, a highly conserved protein co-activating factor of ATGL,[29] while HSL is dispersed in the cytoplasm.[40]

Upon lipolytic stimulation, phosphorylation of both perilipin and HSL by cAMP activated Protein kinase A (PKA) is required. PKA is an enzyme known to participate in the regulation of glycogen, sugar, and lipid metabolism in a wide variety of cells in a cyclic AMP (cAMP) dependent manner. The phosphorylation leads to a stimulated condition where CGI-58 is released from perilipin and HSL is recruited into lipid droplet surface.[19,30,39] Free CGI-58 binds exclusively ATGL[40] recruiting it to the lipid droplet surface via protein-protein interaction and activating its lipolytic functions[34,40] and stimulating the triglyceride hydrolysis and concomitant formation of diacylglycerols by 20-fold at its best.[19,40] The phosphorylation of HSL by PKA affects HSL enzyme activity or its access to substrates.[30] Simultaneous phosphorylation of both perilipin and HSL is necessary for complete hydrolysis of triglycerides[40,42] since only phosphorylated HSL can act on diacylglycerols and hence continue the lipolysis started by ATGL. Hydrolytic activity of HSL against triglycerol or monoacylglycerol is however unaffected by phosphorylation,[41] which is probably one of the reasons for HSL's preference for diacylglycerols. HSL activity can be inactivated by protein phosphatases.[41]

PAT proteins likely affect mast cell lipid droplet hydrolysis. Perilipin 2 is expressed in small amounts in all mammalian cell types analyzed so far[31] and highly expressed in the developing and mature mast cells, where it locates at the circumferential surface of lipid droplets.[29,31,43] PLIN2 shields the triglycerides stored in the lipid core from the activity of cytosolic lipases[19] by controlling the lipolysis catalyzed by Protein kinase A phosphorylated HSL[30] and by regulating the ATGL enzyme activity in a reducing manner[39]. Expression of the PLIN2 gene is itself regulated by the prostaglandins, PGI_2 and PGJ_2 from the COX-2 pathway.[31]

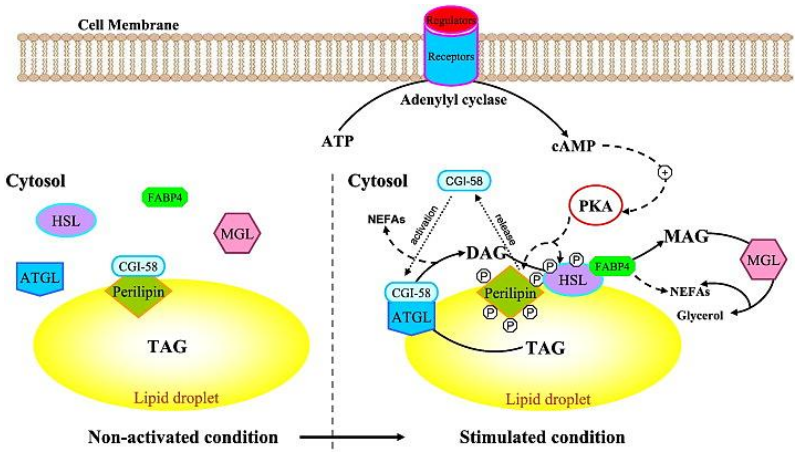


Figure 4: The regulation of HSL and ATGL activity by the PAT protein Perilipin. Phosphorylation of Perilipin and HSL is known to lead to activation of ATGL by CGI-58.[40]

2 Aim of the work

Lipid droplets have been extensively studied in adipocytes and in macrophage foam cells, where the organelles are easy to recognize and visualize, but lipid droplet (LD) biology in mast cells has gained less interest. Although lipid droplets (LDs) have been identified as a site for the conversion of arachidonic acid released from phospholipids by phospholipases into eicosanoids, the mechanism by which arachidonic acid in triglycerides is formed and mobilized has remained mainly undefined.[29] Much more is known about the eicosanoid metabolism of arachidonic acid released from phospholipids by action of Phospholipase A2 (cPLA2). The aim of the study was the elucidation of molecular mechanisms involved in the generation of eicosanoids in human mast cells with particular emphasis on triglyceride-rich lipid droplets as a source and/or site of lipid mediator genesis.

We were interested in finding out which are the enzymes involved in arachidonic acid liberation from triglycerides in lipid droplet core and whether the arachidonic acid from triglycerides in lipid droplets is used for the biosynthesis of eicosanoids, especially the PGD_2. If the lipid-body core triglycerides are the immediate source of arachidonic acid there should be a lipase that is able to respond to cell activation and to mobilize arachidonic acid from triglycerides. We hypothesized that ATGL and/or HSL are putative enzymes in the liberation of arachidonic acid from the lipid droplet triglycerides in mast cells. In addition we were also interested if mast cell lipid droplets are the actual cellular compartments of PGD_2 synthesis. Of all the related enzymes, we were especially interested in the role and presence of HPGDS in lipid droplets.

3 Materials and methods

3.1 Cell culture

Because mast cells do not circulate in peripheral blood and are difficult to obtain and culture from tissue, mature human mast cells were generated according to a well-defined protocol established in our laboratory.[16] For this purpose, $CD34^+$ progenitors were obtained from fresh buffy coats prepared from peripheral blood of healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). The blood sample was transferred into a cell culture bottle containing Ca^{2+}/Mg^{2+} -free PBS and layered on top of Ficoll-Paque (1.77g/l) (Amersham Biosciences 17-1440-03; 500ml). During centrifugation at 800 x g for 30 minutes at room temperature (RT) the Ficoll-Paque fractionated the blood into layers of plasma, buffy coat (the interface containing mononuclear cells), and red blood cells. The isolation of $CD34^+$ cells was carried out with indirect CD34 MicroBead Kit (#130-046-701; MACS®). The buffy coats were washed twice with Ca^{2+}/Mg^{2+} – free PBS and centrifuged at 800 x g for 10 minutes at room temperature and then twice at 200 x g for 10 minutes. The cells were then re-suspended into Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) containing Penicillin Streptomycin mixture. Cell number and viability were determined using a NucleoCounter® NC-200™. The cell suspension was centrifuged at 300 x g for 10 minutes and re-suspended in 400 µl of BIT per 10^8 cells. One hundred microliters of FcR blocking reagent were added and after mixing, the cells were incubated for 15 minutes with 100 µl of CD34-Hapten-antibody per 10^8 total cells. The cells were then washed with 5-10 ml of BIT per 10^8 cells, centrifuged at 300 x g for 10 minutes and re-suspended in 400 µl of the buffer. After this, 100 µl of Anti-Hapten MicroBeads per 10^8 cells were added and the mixture was incubated for 15 minutes. Final washing was done by adding 10 ml of BIT per 10^8 cells and centrifuging at 300 x g for 5 minutes. The cells (up to 10^8) were then re-suspended in 3 ml of BIT, and loaded onto a MACS ® column which was placed in a magnetic field. The eluates were collected by centrifugation (300 x g´s for 5 min), re-suspended in a serum free BIT-medium containing KITL (100 ng/mL) and IL-3, and cultured on 6-well plates. Culturing proceeded under serum-free conditions in Iscove's Modified Dulbecco's Medium (IMDM, Gibco® by Life Technologies) supplemented with BIT 9500 serum substitute (Stem cell Technologies), L-glutamine (2 mM), 2-mercaptoethanol (0.1 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml), human recombinant KITL (100 ng/mL) and different human recombinant cytokines according to a method presented by Lappalainen et al.[16] The weekly incubation schedule can be found as an attachment at

the end of this thesis (Attachment 1). The cells were cultured in a humified incubator at + 37 °C. Cell number and viability were determined from the week two onwards using the NucleoCounter® NC-200™. For optimal cell growth, the cell density was maintained at $5x10^5$cells/ml. The cells chosen for each experiment were maintained i.e. 1ml of BIT-medium was added per 500 00 of re-suspended cells the previous day, and were most often on 6^{th} or 7^{th} week of culture.

3.2 Oil-Red-O staining

Cytospins were prepared in duplicates, 25 000 cells/slide (Cytospin2 SHANDON Instruments). Then slides were fixed in 4% neutral-buffered formalin for 30 seconds, rinsed with running tape water for 10 minutes, incubated for 5 minutes in 100% Propylene glycol and stained for 30 minutes with Oil-Red-O (2.8 g Oil-Red-O, SIGMA), and 400 ml propylene glycol. Then the slides were incubated for 1 minute in 85% Propylene Glycol, rinsed with H2Odd, and stained for 1min in Mayer´s Hematoxylin. Finally, slides were rinsed with tape water and H2Odd before mounting them with Fluorescent Mounting Medium (Dako). Images were captured with Nikon ECLIPSE E600, magnification 40x.

3.3 Basic molecular biological methods

3.3.1 RNA isolation and cDNA synthesis

Total RNA was isolated from the human mast cells using the NucleoSpin®RNA II kit (Macherey Nagel). Isolation of RNA was performed according to the manufactures instructions. All centrifugation steps were carried out at room temperature for 1 min at 11,000 x g. Briefly, mast cells were lysed with 350 µl of Buffer RA1 containing 3.5 µl of β-mercaptoethanol, and RNA was precipitated by adding 350 µl of 70% ethanol. RNA precipitates were loaded on the columns provided. The silica membrane was desalted with 350 µl of MDB (Membrane Desalting Buffer) and on-column DNA digestion was performed for 15 minutes at room temperature using 95 µl of rDNase reaction mixture (10 µl of reconstituted rDNase and 90 µl of Reaction Buffer for rDNase). rDNase was inactivated with 200 µl of Buffer RA2. RNA was washed twice with Buffer RA3. Finally, RNA was eluted with 30 µl of RNase-free H2Odd. RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer.

cDNA was generated from 500 ng of total RNA by First-strand synthesis using random hexamers and M-MLV reverse transcriptase -enzyme according to the manufacturer's instructions (Promega).

Briefly, 3 µl of reaction Mix 1, containing 1 µl of 10mM dNTP mix, 1 µl of Random primers diluted to 200 ng/µl and 1 µl RNase-free water, was added to each sample tube containing 500 ng RNA in a total volume of 11 µl. Samples were incubated at 65° for 5 minutes in a Eppendorf Mastercycler gradient, program JLRT1, and then chilled on ice for 2 minutes. Then, 6 µl of reaction Mix 2 containing, 0.75 µl of RNase-free water, 4 µl of 5X First Strand Buffer, 0.5 µl RNase Inhibitor (40U/ µl), and 0.75 µl of M-MLV RT enzyme (200U/ µl) was added to each RNA/primer mixture. First strand cDNA synthesis was continued according to the following program: 8 minutes at 25° to ensure primer annealing, 55 minutes at 48° for first strand synthesis and 10 minutes at 95° to terminate the reaction. The cDNA samples were stored at -20°.

3.3.2 Quantitative PCR (qPCR)

For qPCR, cDNA was amplified in duplicates using either TaqMan Universal PCR Master Mix (Applied Biosystems) or Power SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific oligonucleotides and fluorogenic TaqMan probes on an ABI PRISM 7500 sequence detector system (Applied Biosystems). Specific oligonucleotides and probes were designed for ATGL, HSL, CGI-58, COX-1, COX-2, HPGDS and GAPDH (Table 1). To compare the expression of the genes chosen, relative quantification was used and data were normalized to the expression of GAPDH. The normalization of qPCR results was done using the Delta-delta CT calculation method, which gives the ratio of the target gene in ones treated sample relative to ones untreated sample by taking $2^{\Delta\Delta CT}$, assuming that the amplification efficiency in each PCR cycle for both the reference control gene and the target gene have been 100%.[44]

Table 1: Primers and probes used in quantitative real-time RT-PCR

Primer or probe	Sequence
ATGL -F*	5' - CAGACGGCGAGAATGTCATT - 3'
ATGL -R#	5' - AAATGCCACCATCCACGTAG- 3',
HSL-F	5' - GAGTGCTAGGCACATAGTCCCCTCC- 3'
HSL-R	5' - GCGGGGTTATAGGCCTCTGGTG- 3'
hsCGI58_F2	5' - AGCCTTGGGGTTTCCCTGAACG- 3'
hsCGI58_R2	5' -AATCAGGCCTTAAACGCTGCACT- 3'
COX-1 -F	5' - CACAGTGCGCTCCAACCTTA- 3'
COX-1 -R	5' - TGGAGAAAGACTCCCAGCTGA- 3'
COX-1 probe	5' - CTTATCCCCAGTCCCCCCACCTACAACTC- 3'
COX-2 -F	5' - GGCGCAGTTTACGCTGTCTAG- 3'
COX-2 -R	5' -CGAGGGCCAGCTTTCAC- 3'
COX-2 probe	information unavailable
HPGDS-F	5' -ATGCGCCTCATCTTATGCAAG- 3'
HPGDS-R	5' -GGTTGTCTAACAGGTCAGGCT- 3'
GAPDH-F	5' -GTCAACGGATTTGGTCGTATTGG- 3'
GAPDH-R	5' -GGCAACAATATCCACTTTACCAGAGT- 3'
GAPDH probe	5' - TGGTCACCAGGGCTGCTT- 3'
* forwar primer	
# reverse primer	

3.4 Basic molecular biochemical methods

3.4.1.1 Preparation of protein lysates

For the preparation of total cell lysates, mast cells were washed twice with PBS, lysed in ice-cold cell lysis buffer (25 mM Tris/HCl pH 7.4, 150mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol) containing complete protease inhibitor cocktail (Roche). Protein concentrations were determined by using BCA protein kit from Pierce (Pierce ® BCA Protein Assay Kit, Thermo Scientific Prod # 23225, Lot # MA152117).

3.4.1.2 Western blot analysis

Proteins were separated by SDS-PAGE (10 % gels; 1,5 mm) under reducing condition, and then transferred onto a nitrocellulose membrane by semi-dry blotting for 1h at 200 mAmp using 1x transfer buffer (10x Transfer buffer for Wet Blot or Semi-Dry Blot 250mM Tris, 1.92M Glycine, in 1:10 dilution) (Hybond-C Extra; Amersham Biosciences). Non-specific binding sites were blocked with 5% non-fat dry milk in 1x TBS-T buffer (150 mM NaCl, 10 mM Tris, 0.1% Tween-20, pH 8) for 1 hour at room temperature. Immunodetection was performed using mouse anti-human HPGDS

(0.1 µg/ml; MAB6487; R&D Systems) followed by incubation, according to the primary antibody, with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (1:2000 dilution; 0447; DAKO). The GAPDH antibody (Monoclonal α-GAPDH mouse IgM isotype, cat No. G8795 SIGMA-ALDRICH) was used in 1:2000 dilutions followed by incubation and immunodetection with HRP-conjugated goat α-mouse IgG in 1: 25000 dilutions (DAKO). The signals were detected using an enhanced chemiluminescence method (PIERCE).

3.5 siRNA transfection

ATGL and HSL were specifically knocked down by small interfering RNAs (siRNAs) purchased from QIAGEN (for product details see table 2). Mature human mast cells were freshly maintained 24 h prior transfection. For siRNA transfection mast cells were collected by centrifugation at 300 x g for 5 minutes, and cell density was adjusted to 250 000 cells/100 µl pre-conditioned medium. The remaining pre-conditioned medium was collected and stored at $+4^{o}$ C. The cells were seeded on 24-well plates at a density of 2.5 x 10^{5} cells/100 µl/well. RNA:lipid complexes were generated as follows: the siRNAs (molar ratio 1:1:1:1; total of 100nM) were mixed with pre-conditioned medium and HiPerfect transfection reagent. The siRNA:lipid complexes were allowed to form during a 10-minute incubation at room temperature. siRNAs for human ATGL (GeneSolution siRNA, cat.No. SI03019310; SI03019611; SI05654397; SI05654404, QIAGEN), and human HSL (GeneSolution siRNA, cat. No., SI00470834; SI04159904 ; SI04208659 ; SI04280346 , QIAGEN) are indicated in table 2. Cells were transfected with siRNAs or 100nM Allstar negative control siRNA AF488. Twenty hours after transfection, the cells were subjected to immunological activation as described below.

Table 2: siRNAs used in siRNA transfection of human mast cells

siRNA	Product name	Coding sequence
ATGL	Hs_PNPLA2_5	CAAGTTCATTGAGGTATCTAA
ATGL	Hs_PNPLA2_6	GACGGCGAGAATGTCATTATA
ATGL	Hs_LOC100507839_2	AAGTTCATTGAGGTATCTAAA
ATGL	Hs_LOC100507839_3	CAACACCAGCATCCAGTTCAA
HSL	Hs_LIPE_3	CAGCAGCCTGATAAAGTCCAA
HSL	Hs_LIPE_5	CCGCTAGAGCCTGGGCCAGAA
HSL	Hs_LIPE_6	CCCTCCGATGTCAACTTCTTA
HSL	Hs_LIPE_7	CAGGAACTATCCATACAGCGA

3.6 Mast cell activation

For immunological activation via IgE receptor cross-linking, mast cells were first sensitized for 3 hours with human IgE (1µg/ml, DiaTec), and then activated by incubation in the presence of polyclonal rabbit anti-human IgE (1µg/ml, Millipore) for different periods of time. Mast cells incubated for 3 hours with 1µg/ml IgE alone were used as non-activated controls.

3.7 PGD_2 MOX EIA

The quantities of PGD_2 released from mast cells into the culture medium were analyzed by using commercial enzyme immunoassays (Prostaglandin D_2-MOX EIA Kit, Cayman Chemicals) according to the manufacturer's protocols. Briefly, the assay is based on the conversion of PGD_2 to a more stable MOX-derivative by methoxylamine hydrochloride treatment. Preparation of the methoximated PGD_2 standards was performed according to the assay protocol. Standards, controls and samples were prepared in duplicates according to the protocol in the kit. NSB controls were prepared by mixing 105 µl of BIT, EIA buffer and Tracer each and B0 control by mixing 52.5 µl of BIT and EIA buffer with 105 µl of Tracer and Antiserum. Standards and samples were prepared by mixing 105 µl of sample or standard with equivalent amounts of Tracer and Antiserum. Blank and TA control wells were kept empty; the well plate was sealed and then incubated over night at 4ºC. The ELISA development was done by washing all wells with 5 times 200 µl EIA Wash-buffer after which 200 µl Ellmann's reagent was added to each well. The TA wells received 5 µl of Tracer as well. The plate was sealed and incubated 1 h at room temperature after which the absorbance at 450 nm, Scale: 0.000A-2.000A: Absorbance Scale (for photometry) was read with PerkinElmer

precisely 1420 Multilabel counter VICTOR ^{3}™ and with Wallac 1420 Manager Software. In this reading, the more intense the yellow signal is, the less free PGD_2 -MOX is present.

3.8 Preparation of FA:BSA complexes

Fatty acid:BSA complexes were prepared as described by Dichlberger et al.[29] Briefly, stock solutions of a fatty acid sodium salt arachidonate or oleate (Sigma, A8798) were combined with a stock solution of fatty acid-free BSA (Sigma, A6003) at a molar ratio of 6:1 (fatty acid:BSA) under nitrogen at 37°C for 15 min. All stock solutions were prepared in LDL buffer (pH 7.4; 150mM NaCl; 0,24mM EDTA). Measurement of fatty acid concentration was carried out using the NEFA analysis kit from WAKO. Fatty acid: BSA complexes were used for induction of lipid droplet formation in human mast cells.

3.9 Immunofluorescence stainings

Mast cell lipid droplets were induced by exogenous treatment with oleic acid or arachidonic acid complexed to BSA (250 µM final fatty acid concentration) for 18 hours. The following day the induced cells were activated as mentioned before. The cytospins were prepared in duplicates and cells fixed with 4% PFA 0.025% GA in Ca^{2+}/Mg^{2+} –free PBS for 20 min in room temperature. The slides were then washed four times 5 minutes with PBS on a shaker. Non-specific binding sites were blocked with 3% goat serum 0.1% Saponin (Fluka, Lot # 1370302) in PBS (pH 7.4) containing 0.2M glycine (SIGMA, product code 101175631 50046-1KG, Lot# BCBG8981V). Primary and secondary antibodies were both diluted in 3% goat serum 0.1% saponin in PBS. The primary ATGL antibody (Cell Signalling TECNOLOGY, # 2138) was used in 1:100 dilutions and the primary HPGDS Antibody (R&D SYSTEMS, HPGDS ANTIBODY Monoclonal Mouse IgG_1 Clone#735301 Cat. No. MAB6487, Lot# CFWO0111081) in 1:200 dilutions. Mouse IgG1 Negative /Isotype control (AbD Serotec, MCA928, 0.1mg/ml) was used as negative control. ALEXA 594 goat α-rabbit IgG (Invitrogen™ Life Technologies, Molecular Probes® A11080, Lot# 459560; dilution 1:500) or Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) (Invitrogen™ Life Technologies) were used as the secondary antibody. The primary antibodies were incubated overnight at +4°C and the secondary antibodies for one hour at room temperature. Finally, the slides were washed 3 times with Ca^{2+}/Mg –free PBS for 5 minutes, and counterstained with DAPI. The lipid droplet content was assessed by adding BODIPY fluorescent dye (10 µg /ml) simultaneously with the secondary antibody. The slides were mounted using Fluorescent Mounting Medium from Dako and stored

protected from light. Images were captured with Nikon ECLIPSE E600 (original magnification, 40x).

4 Results

For the generation of mature and functional human mast cells, $CD34^+$ peripheral blood progenitors were isolated from different human donors and differentiated into mature mast cells under serum-free conditions for up to 6 to 8 weeks. The presence of cytoplasmic lipid droplets was visualized by Oil Red O staining (Fig. 5). The results confirmed the earlier reported observation made in our laboratory that not just mature mast cells isolated from human lungs[23] but also the in vitro-generated human mast cells derived from peripheral blood progenitor cells contain lipid droplets (LDs) in their cytosol.[29] Already at week 5 of culture, almost all mast cells stained positively for lipid droplets; however, in contrast to the lung mast cells,[23] the lipid droplets were located not mainly around the nucleus, but were evenly distributed in the cytoplasm (Fig. 5). The lipid droplets were of different sizes, a finding we previously made while studying cultured mast cells under culture conditions identical to those described here[29].

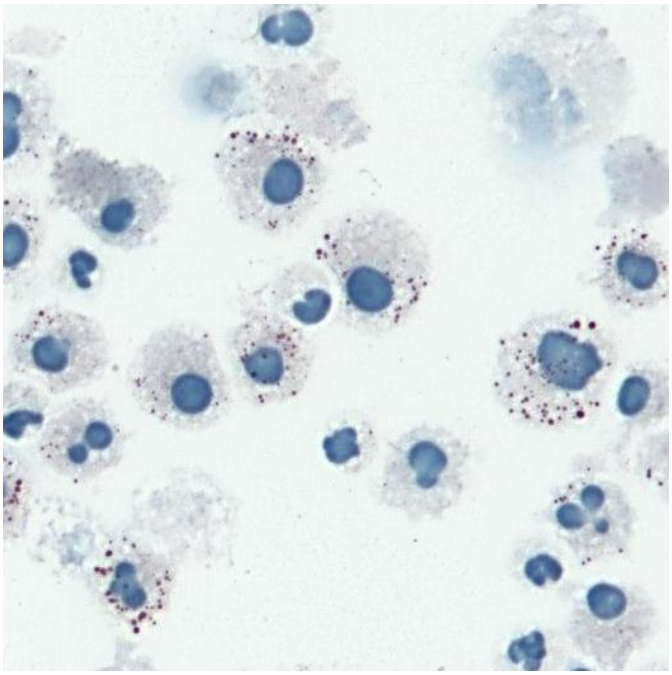


Figure 5: Identification of human mast cell lipid droplets with light microscopy on their 5th week of culture. $CD34^+$ peripheral blood progenitors were isolated from different donors and cultured under serum-free conditions to generate mature mast cells as described in Materials and methods. The cells were stained with Oil Red O and Hematoxylin in week 5 of culture. Mast cells from a single donor are shown at magnification 40x.

As we were interested in the release of arachidonic acid from lipid droplet triglycerides for the purpose of prostaglandin D2 (PGD_2) generation upon mast cell activation, we first analyzed the gene expression of certain enzymes involved in this process. The biosynthesis of PGD_2 is well-known to proceed via the COX pathway.[21-23] Cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) catalyze the enzymatic reaction from arachidonic acid to prostaglandin $PG(H)_2$, a common precursor for prostaglandins,[14,21-23] which is further metabolized by HPGDS to generate PGD_2[22]. Potent candidates for enzymes releasing arachidonic acid from triglycerides are ATGL and HSL. qPCR analysis was used to measure transcript levels of ATGL, CGI-58, HSL, COX-1, COX-2 and HPGDS at different time points after IgE-mediated mast cell activation. For this purpose mast cell RNA was purified using the NucleoSpin®RNA II kit, the RNA was reverse transcribed into cDNA, and the cDNA was amplified using qPCR and GAPDH as reference gene for data normalization.

The transcript levels of ATGL did not change at 0.5 and 1 h post-activation, but showed an up-regulation 2 h after cellular activation (Fig. 6a). CGI-58, the co-activating factor of ATGL, showed a slight down regulation of mRNA levels upon mast cell activation, but displayed similar levels at 2 h post-activation as observed in resting cells (Fig. 6b). HSL transcript levels clearly dropped upon mast cell activation and stayed at a lower level compared to resting cells (Fig. 6c) Moreover, we analyzed the gene expression of COX-1, COX-2, and HPGDS, which are involved in the generation of PGD_2. As demonstrated in figures 6d and 6f, neither COX-1 nor HPGDS showed any significant changes in mRNA levels upon mast cell activation. In contrast, COX-2 mRNA levels increased dramatically by about 40 fold at 1 h post-activation and declined to resting levels at 2 h after mast cell activation (Fig. 6e). Since LPGDS does not play any major role in mast cells, analysis of this type of Prostaglandin D synthase was not included in this study.

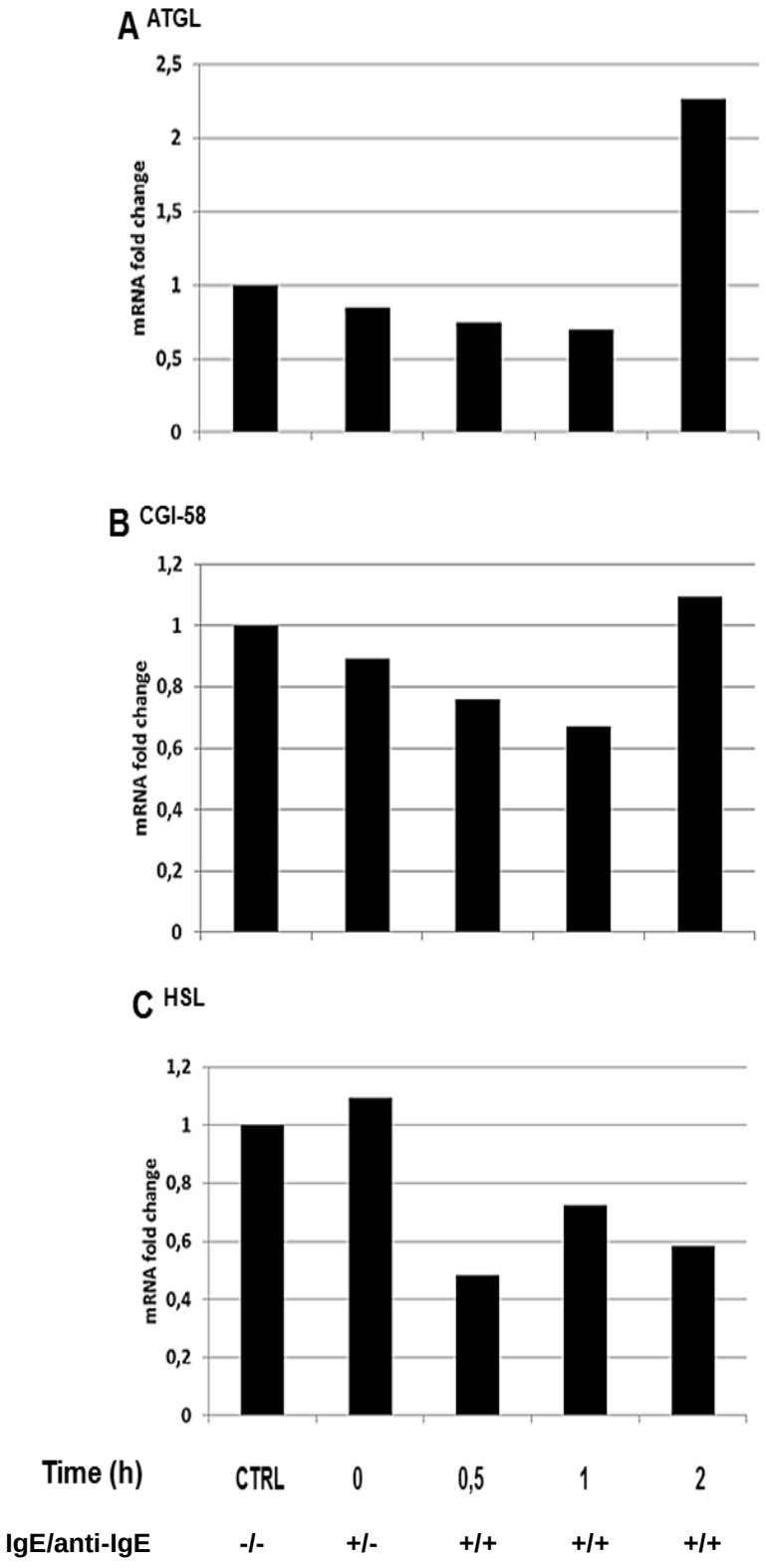


Figure 6 a-c: Expression of ATGL, CGI-58 and HSL in sensitized and activated human mast cells. Mature human mast cells were activated as described in Material and Methods. The expression levels of ATGL, CGI-58 and HSL were determined using qPCR. For data normalization, GAPDH was used as endogenous control. Data with cells derived from one representative donor is shown

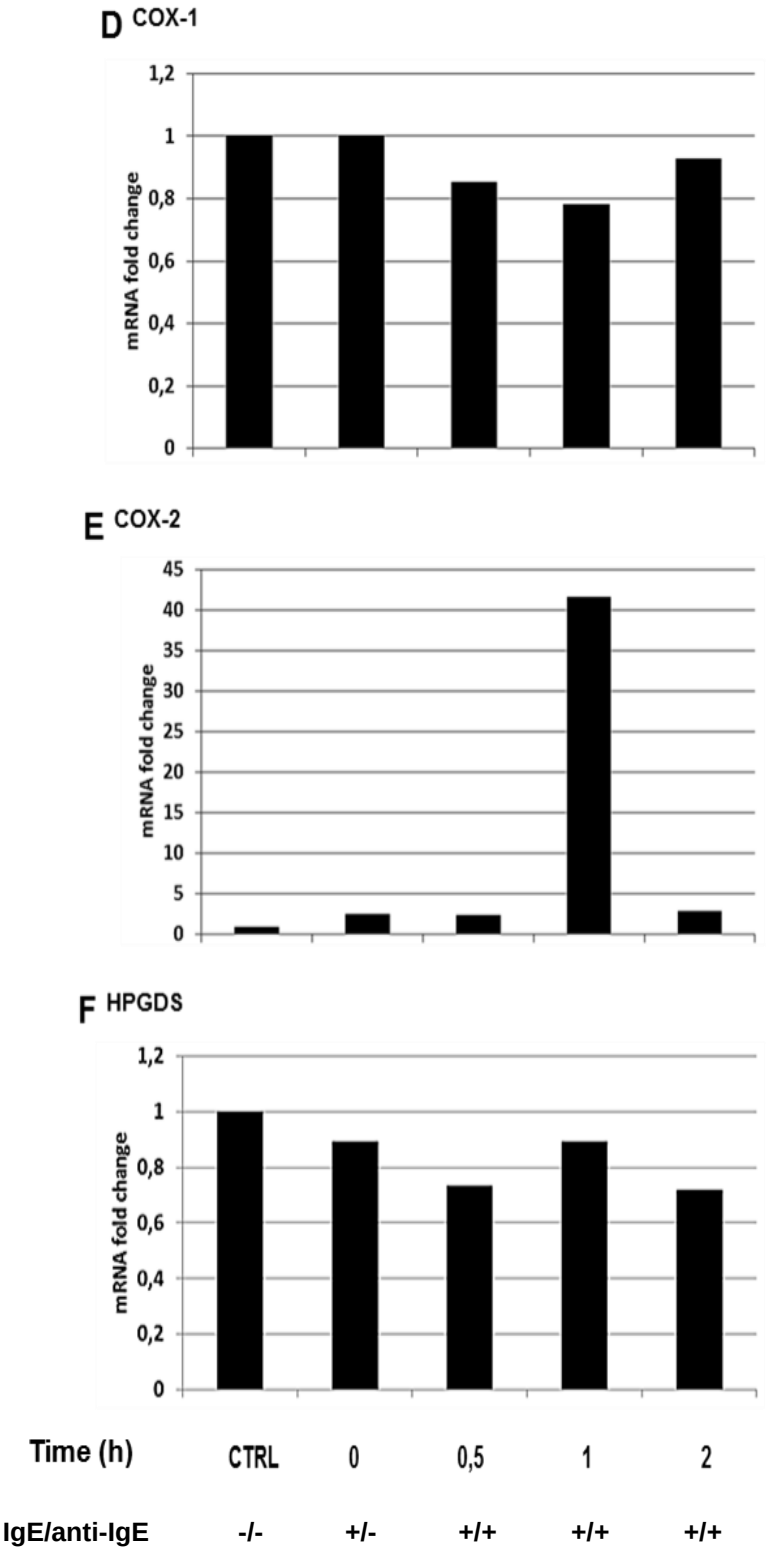


Figure 6 d-f: Expression of COX-1, COX-2 and HPGDS in sensitized and activated human mast cells. Mature human mast cells were activated as described in Material and Methods. The expression levels of COX-1, COX-2 and HPGDS were determined using qPCR with GAPDH as endogenous control. Data with cells derived from one representative donor is shown

Next, we analyzed the amount of PGD_2 released from activated mast cells. The amount of PGD_2 released from activated mast cells into the culture medium was measured by a specific ELISA-protocol, as described in Materials and Methods. The increase of PGD_2 was immediate and most prominent after one hour of activation, and slowly decreased to basal levels at 48 h post-activation (Fig. 7).

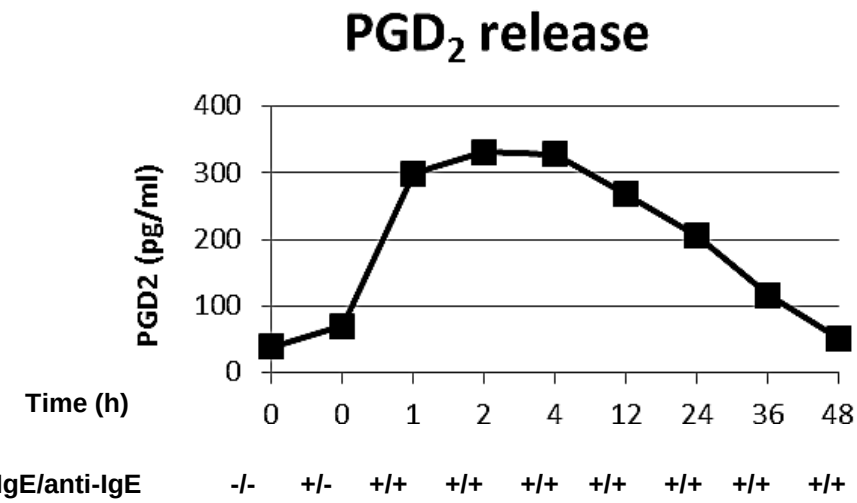


Figure: 7: PGD_2 release from activated human mast cells. Mast cells were immunologically (IgE) activated as described in Materials and Methods. PGD_2 concentrations (pg/ml) were measured at the indicated time points after mast cell activation with a PGD_2 mox immunoassay. Data of one representative donor is shown.

In order to study whether silencing the gene expression of ATGL and/or HSL would affect the quantity of PGD_2 released by activated mast cells, siRNA transfections were performed. The siRNA method used interferes with the transcription-translation -step silencing the messenger RNA (mRNA) levels of the gene in question. Since mast cell activation is known to lead into an increase in the amount of PGD_2 released, we wanted to test the effect of ATGL- and HSL-knockdowns on an early phase (30 to 120 minutes after activation) of PGD_2 release. The quantities of PGD_2 released from ATGL-silenced cells were not different from those in the siRNA control-treated cells (Fig. 8). In contrast, knockdown of HSL showed a clear reduction in PGD_2 release compared to control cells (Fig. 8). Although no effect was observed regarding the quantity of PGD_2 released by ATGL-silenced cells, the knockdown efficiency of ATGL was successful, the ATGL mRNA levels being reduced by about 85% compared to control cells as demonstrated by qPCR (Fig. 9). The ATGL co-activator CGI-58 is known to enhance the activity of ATGL, and knocking down either ATGL or HSL had no effect on the expression of CGI-58 (Fig. 10). Silencing of ATGL or HSL had no significant effect on each other's mRNA expression levels (Fig. 9 and Fig. 11).

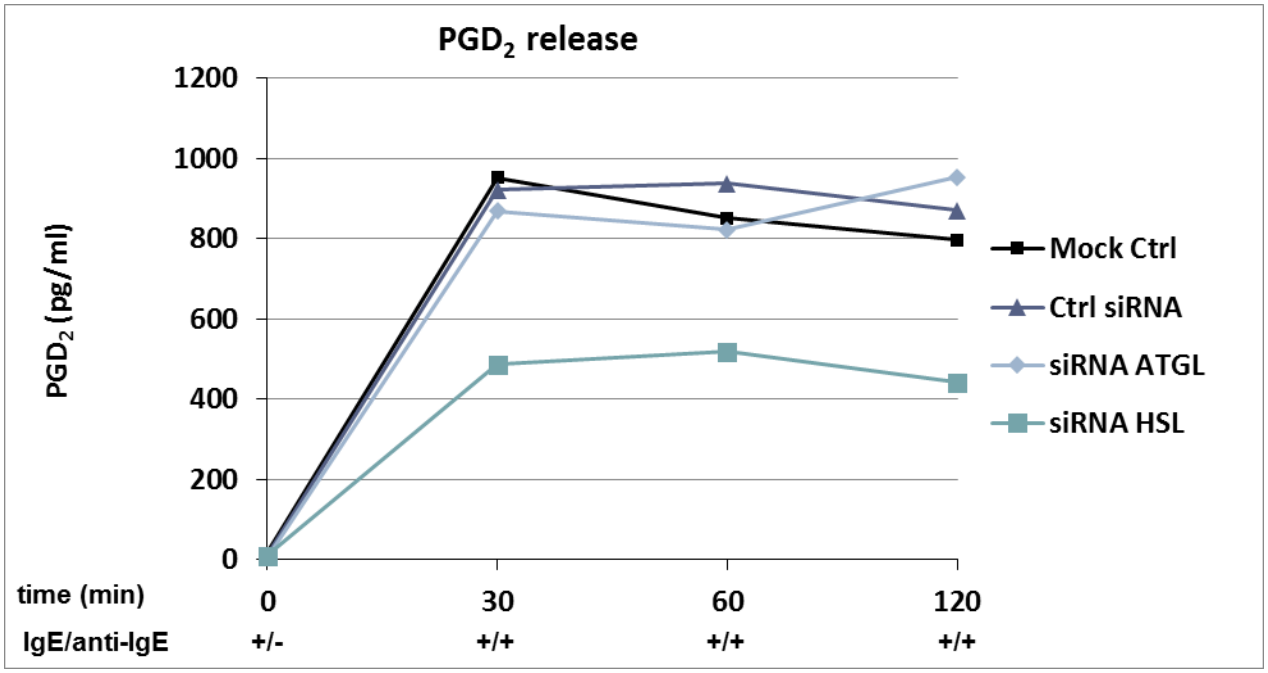


Figure 8: Early phase PGD_2 release from activated human mast cells after ATGL and HSL siRNA knockdowns. Mast cells were activated and the siRNA transfection was performed as described in Materials and Methods. PGD_2 concentrations (pg/ml) were measured at the indicated time points after mast cell activation with a PGD_2 mox immunoassay. Data of one representative donor is shown.

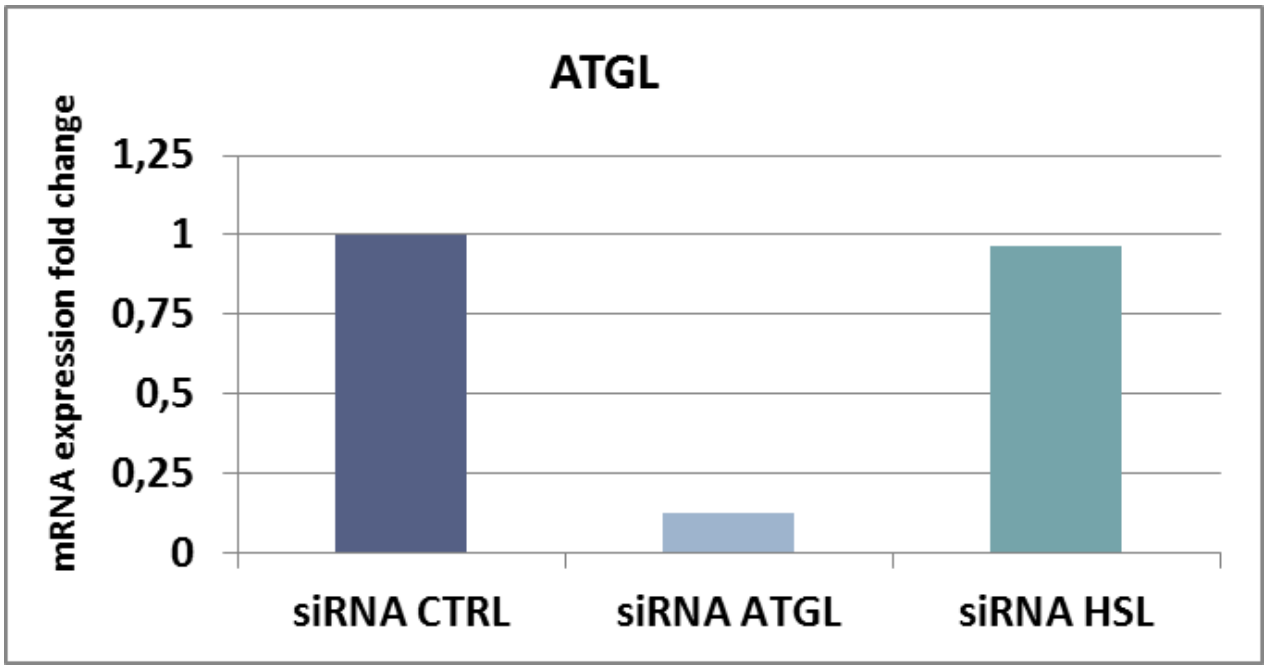


Figure 9: The effect of ATGL and HSL knockdowns by siRNAs for investigating the effect on ATGL expression in activated mast cells. Human mast cells were transfected with siRNAs targeting ATGL (100nM) or HSL (100nM) for 20 h at 37°C. Control siRNA-transfected cells were used as controls. ATGL and HSL mRNA levels were analyzed by qPCR. For data normalization, GAPDH was used as endogenous control. Transcript levels are shown as fold changes, and represent data obtained with cells derived from a single donor.

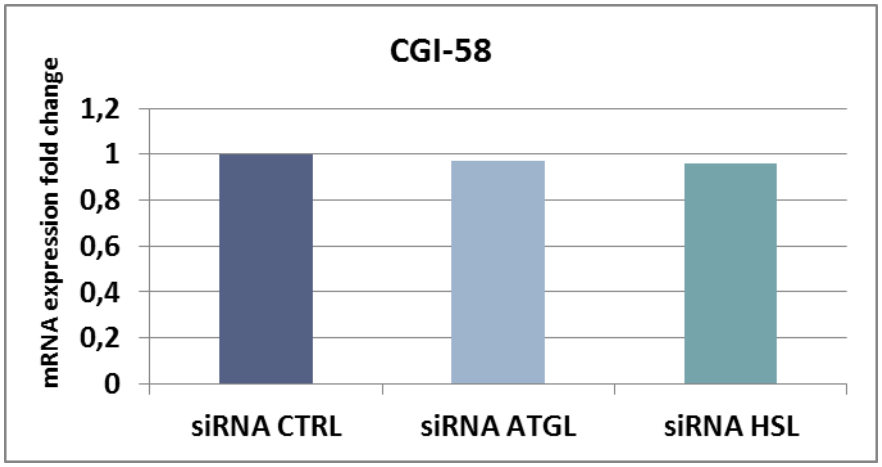


Figure 10: The effect of ATGL and HSL knockdowns by siRNAs for investigating the effect on CGI-58 expression in activated mast cells. Human mast cells were transfected with siRNAs targeting ATGL (100nM) or HSL (100nM) for 20 h at 37°C. Control siRNA-transfected cells were used as controls. ATGL and HSL mRNA levels were analyzed by qPCR. For data normalization, GAPDH was used as endogenous control. Transcript levels are shown as fold changes, and represent data obtained with cells derived from a single donor.

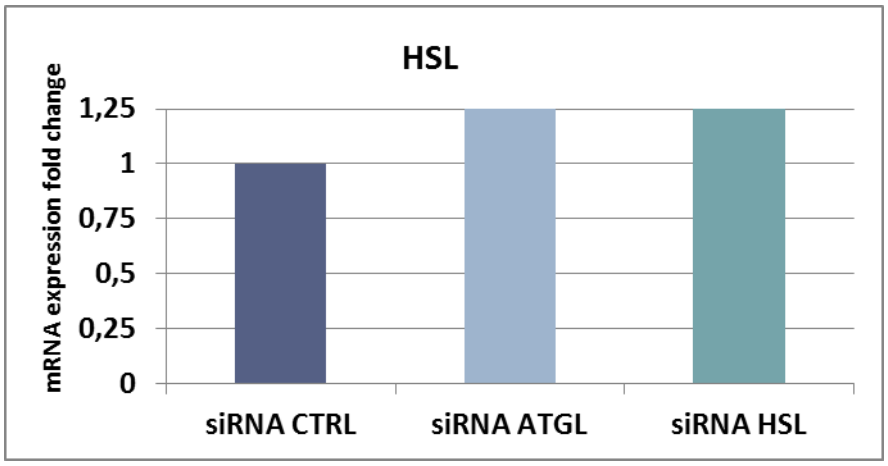


Figure 11: The effect of ATGL and HSL knockdowns by siRNAs for investigating the effect on HSL expression in activated mast cells. Human mast cells were transfected with siRNAs targeting ATGL (100nM) or HSL (100nM) for 20 h at 37°C. Control siRNA-transfected cells were used as controls. ATGL and HSL mRNA levels were analyzed by qPCR. For data normalization, GAPDH was used as endogenous control. Transcript levels are shown as fold changes, and represent data obtained with cells derived from a single donor.

The amount of PGD_2 released during the early phase was further tested with an ATGL-HSL –double knockdown experiment (Fig. 12). When both ATGL and HSL were knocked down, the quantity of PGD_2 released did not show any significant reduction compared to siRNA control-treated cells.

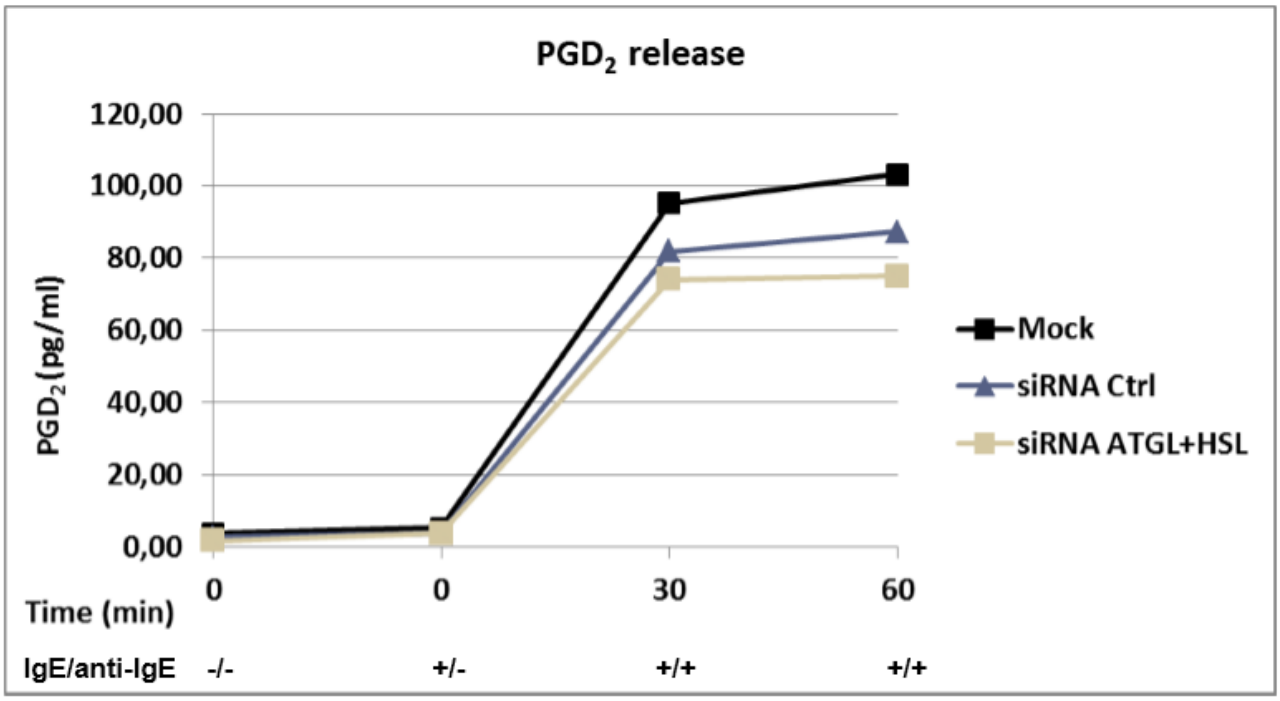


Figure 12: The effect of ATGL and HSL double knockdown on early phase of PGD_2 release. Mast cells were activated and the siRNA transfection was performed as described in Materials and Methods. PGD_2 concentrations (pg/ml) were measured with a PGD_2 mox immunoassay at the indicated time points after mast cell sensitizing and activation. Data of one representative donor is shown.

Since no effects on the release of PGD_2 were observed in ATGL single-silenced or ATGL-HSL –double-silenced mast cells during the early mediator release, we analyzed the immediate (5 min and 15 min) response of mediator release upon mast cell activation. As shown in Fig. 13, the release of PGD_2 was reduced in HSL-silenced mast cells and even more pronounced in ATGL single-silenced cells at 5 and 15 minutes after mast cell activation compared to control cells. By far the strongest reduction in the acute PGD_2 release was seen with the ATGL+HSL –double knockdown and the double knockdown of ATGL and HSL reduced the quantity of PGD_2 released more than either of the two alone. Taken together, the quantity of PGD_2 released from mast cells under silencing conditions was higher after 15 than 5 minutes of activation. Meaning the silencing was stronger after 5 minutes than after 15 minutes.

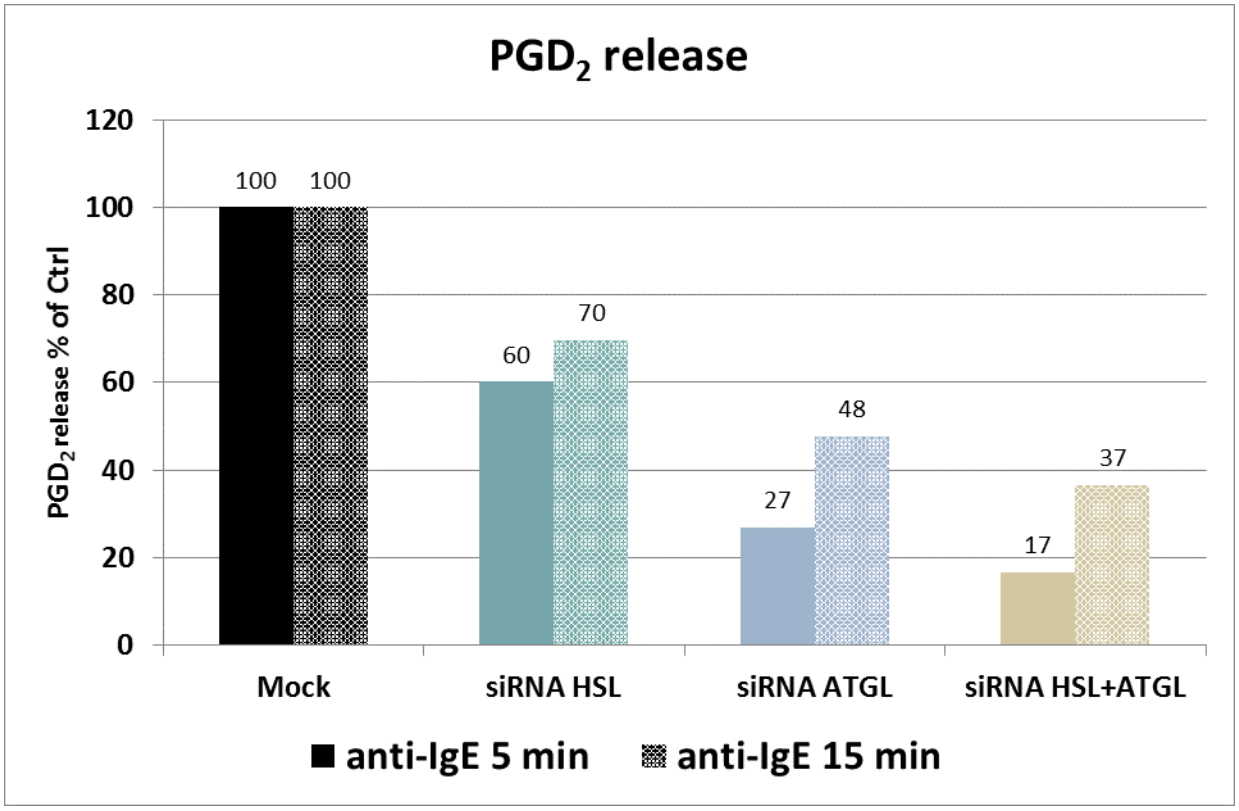


Figure 13: The effect HSL, ATGL and HSL-ATGL -double knockdown on acute PGD_2 release from activated mast cells. Mast cells were activated and the siRNA transfection was performed as described in Materials and Methods. PGD_2 concentrations (pg/ml) were measured at the indicated time points after mast cell activation with a PGD_2 mox immunoassay. Data with cells derived from one representative donor is shown.

In ATGL-silenced and ATGL+HSL-silenced cells, the knockdown efficiency of ATGL was successful and ATGL mRNA levels were somewhat reduced compared to control cells as demonstrated by qPCR (Fig. 14). HSL knockdown had a minimal, but non-significant up-regulating effect on ATGL expression (Fig. 14). Moreover, HSL, ATGL and HSL+ATGL knockdowns had minimal non-significant up-regulating effects on HSL expression (Fig. 15). All the qPCR data was normalized to GAPDH.

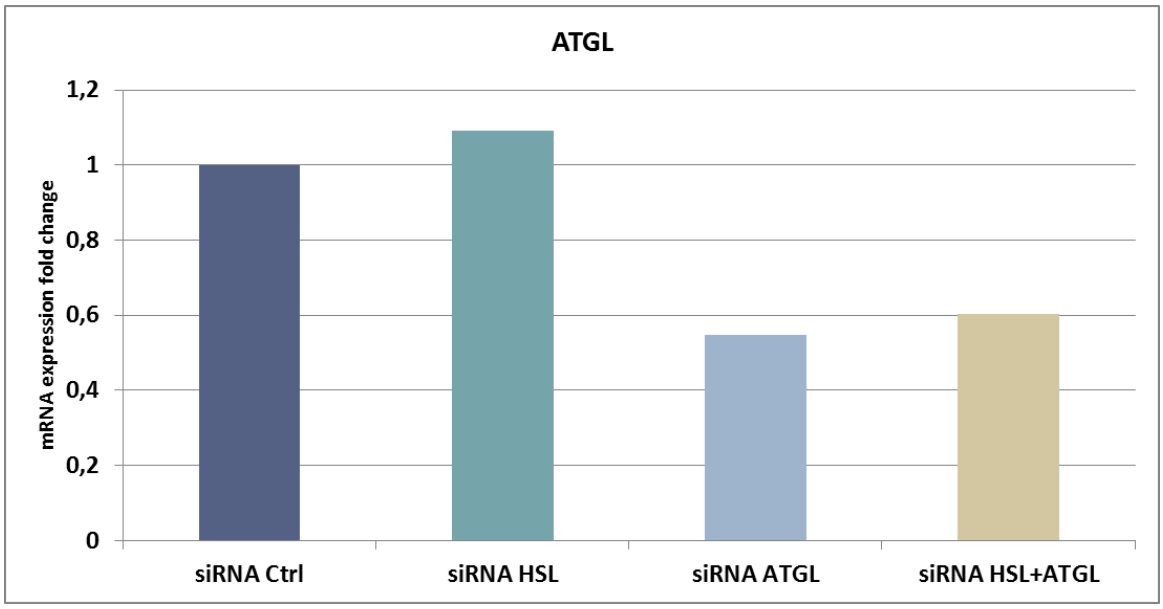


Figure 14: The effect of ATGL+HSL -double knockdown by siRNAs for investigating the effect on ATGL expression in activated mast cells. Human mast cells were transfected with siRNAs targeting ATGL (100nM), HSL (100nM) or ATGL+HSL for 20 h at 37°C. Control siRNA-transfected cells were used as controls. ATGL and HSL mRNA levels were analyzed by qPCR. For data normalization, GAPDH was used as endogenous control. Transcript levels are shown as fold changes and represent data obtained with cells derived from a single donor.

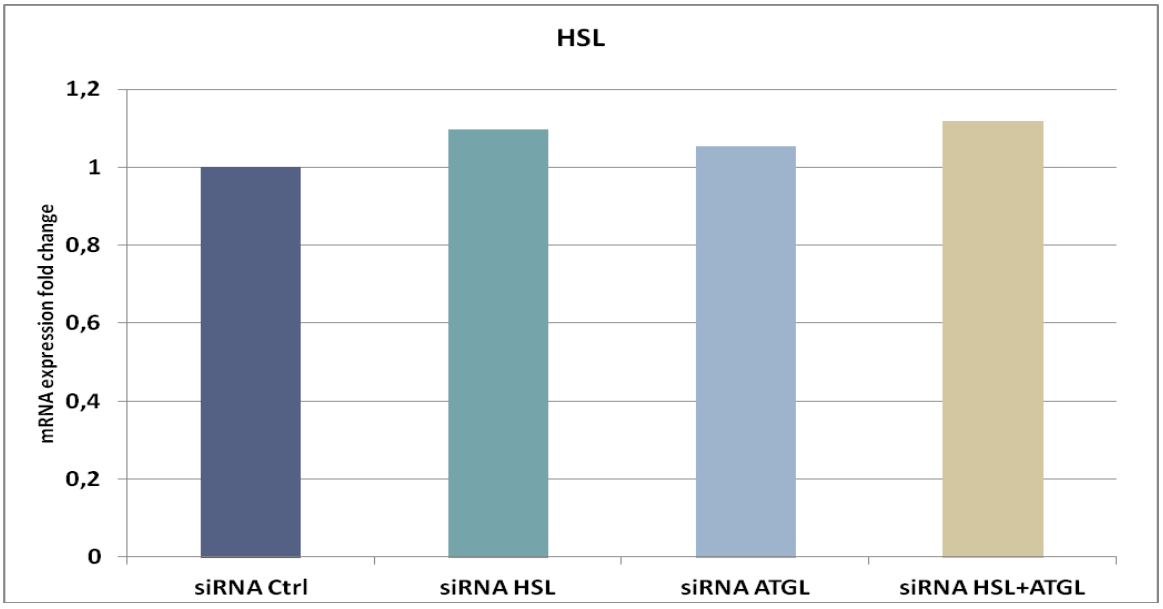


Figure 15: The effect of ATGL+HSL -double knockdown by siRNAs for investigating the effect on HSL expression in activated mast cells at their 11th week of culture. Human mast cells were transfected with siRNAs targeting ATGL (100nM), HSL (100nM) or ATGL+HSL for 20 h at 37°C. Control siRNA-transfected cells were used as controls. ATGL and HSL mRNA levels were analyzed by qPCR. For data normalization, GAPDH was used as endogenous control. Transcript levels are shown as fold changes and represent data obtained with cells derived from a single donor.

In order to obtain information about the intracellular localization of the enzymes of interest, immunofluorescence microscopy was used to assess the localization and endogenous expression of ATGL in human mast cells. Staining with the ATGL antibody revealed that ATGL was widely dispersed in the mast cells (Fig.16a).The size of cytosolic lipid droplets was enhanced by exogenous fatty acid treatment, but only a few lipid droplets were present (Fig. 16 b) and they stained green with BODIPY 493/503 (Fig. 16 c).

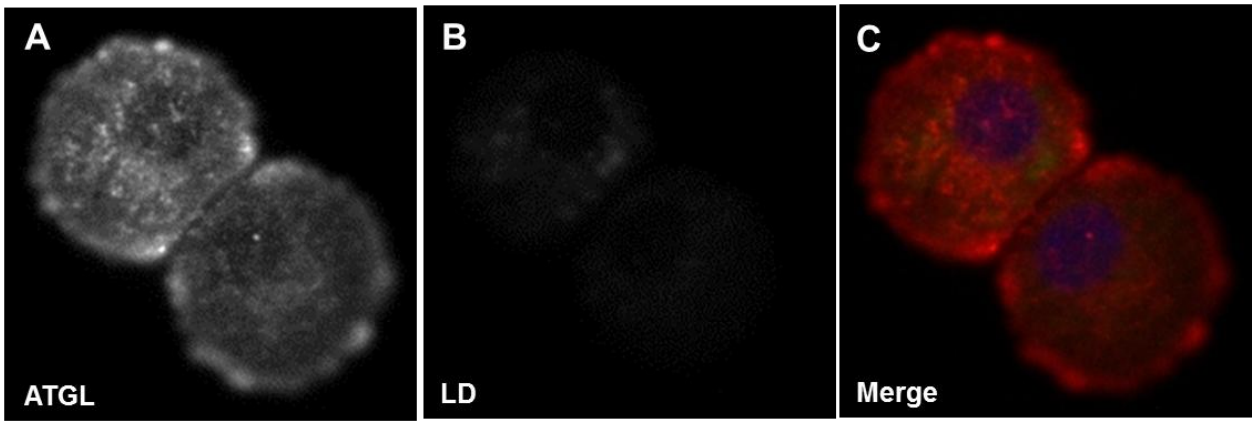


Figure 16: Intracellular localization of human mast cell ATGL. For the analysis of intracellular localization of ATGL, immunofluorescence staining of ATGL in non-activated mast cells on 13th week of culture was performed. The cells were induced with 250 mmol of oleic acid for 18 to 20 hours to increase their lipid droplet (LD) production. The primary ATGL antibody was probed with Alexa Fluor® 594-conjugated goat α-rabbit IgG (red). The cells were stained for lipid droplets (LD) with DOBIPY 493/503 (green), and nuclei were counterstained with DAPI (blue). Merged image is shown in the right-hand panel. Data with cells derived from one representative donor is shown.

Moreover, mast cells were also stained for the cellular distribution of HPGDS under resting and activated conditions. In the resting Ctrl (Fig. 17a, b, c) and IgE control cells (Fig. 17d, e, f) the specific staining for HPGDS was primarily visible in the nuclear and cytosolic area (Fig17a, d). Lipid droplets were clearly visible as homogenously stained round-shaped entities (Fig. 17b, c) staining positive with BODIPY493/503. The same observation was made in mast cells 15 min after IgE-mediated activation (Fig.17g, i). Both, the nucleus and the cytoplasm showed a positive staining for HPGDS. Interestingly, 30 min after mast cell activation, only cytosolic staining around the nucleus was observed for HPGDS as shown in Fig.17j and l. A strong HPGDS-positive cytosolic, but very weak nuclear staining was observed 60 min post-activation (Fig. 17m, o). In the negative controls treated with mouse isotype IgGs specific binding of the secondary antibody was observed.

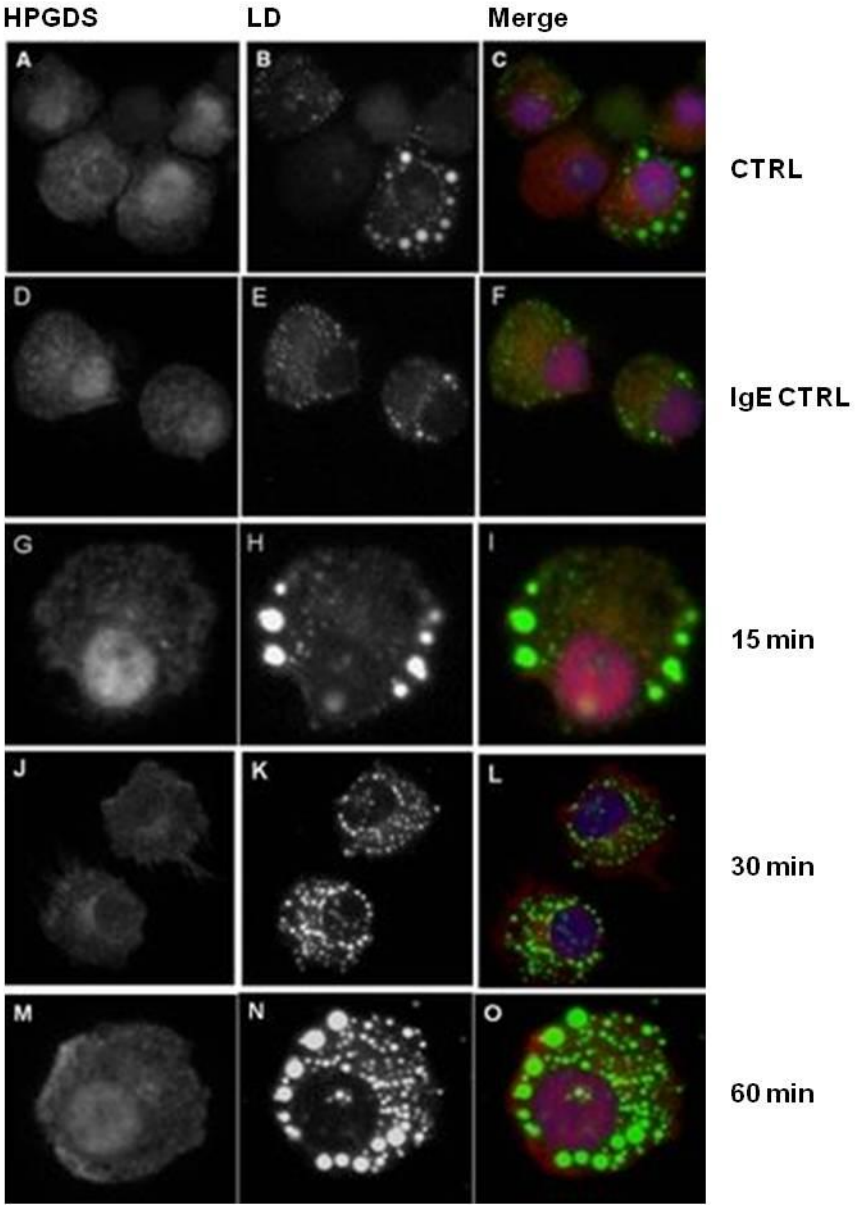


Figure 17: Intracellular localization of human mast cell HPGDS. Mast cell lipid droplets were induced by exogenous treatment with arachidonic acid complexed to BSA (250 µM final fatty acid concentration) for 18 hours. Localization of mast cell HPGDS was investigated by immunofluorescence staining. Mast cells were stained under resting and activated conditions at different time points (15, 30, and 60 min post-activation). The primary HPGDS antibody was probed with Alexa Fluor® 594-conjugated Goat Anti-Mouse IgG (red). Lipid droplets (LD) were stained with BODIPY 493/503 (green) and nuclei were counterstained with DAPI (blue). Merged images are shown in the right-hand panels. Data with cells derived from one representative donor is shown

Finally, the protein expression of HPGDS was analyzed in human mast cells by Western blotting. A 23 kDa immuno-reactive band, representing HPGDS, was detected in mast cells under resting and activated conditions (Fig 18). The expression of HPGDS was weak in resting cells, but clearly increased upon activation of the cells at all of the time points (Fig 18 lanes 1, 3-6, respectively). The level of HPGDS expression stayed the same over the entire period (up to 4 h after mast cell activation, lane 6). GAPDH was used as a loading control and displayed equal expression under both resting and activated conditions (Fig. 18 lower part).

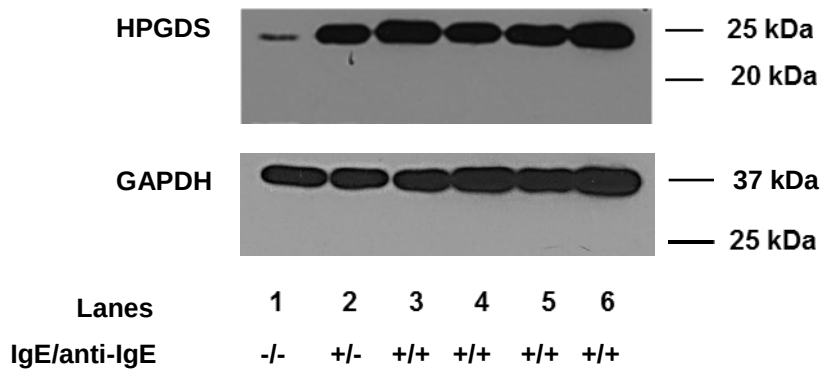


Figure 18: HPGDS protein in CD 34+ derived human mast cells. Total mast cell protein lysates were separated under reducing conditions by 12% SDS-PAGE, blotted onto nitrocellulose membrane and probed with mouse anti-human HPGDS (0.1 µg/ml). GAPDH was used as a loading control. Lanes: 1. CTRL, 2. IgE CTRL (3 h at +37° C), 3. αIgE 30 min, 4. αIgE 1 h, 5. αIgE 2 h, 6. αIgE 4 h. Data with cells derived from one representative donor is shown.

5 Discussion

First observations that mast cell lipid droplets might serve as sites of eicosanoid biosynthesis were published already in 1980s by Dvorak and co-workers, when they found large quantities of arachidonic acid in cytoplasmic lipid droplets of human lung mast cells.[23] The research group came to the conclusion that this myriad of arachidonic acid in lipid droplets must have a critical function in mast cell biology. Later in 1992, an observation was published which demonstrated the presence of prostaglandin endoperoxide (PGH) synthase (cyclooxygenase) in lipid droplets of human lung mast cells.[45] Another interesting observation was reported by Triggiani et al.[46] who compared the total cellular content of arachidonic acid among different types of immune cells. They published that human lung mast cells had the highest arachidonic acid content with almost 50% of arachidonic acid being incorporated into neutral lipids, and that within the neutral lipids over 80% were found to be esterified into triglycerides. Altogether, these interesting findings support the idea that mast cell lipid droplets are cellular organelles in which lipid mediator generation may take place.

In this study we focused on the role of ATGL and HSL in human mast cells as putative candidates for releasing arachidonic acid from triglycerides of lipid droplets for the generation of PGD_2. Exogenous arachidonic acid has been shown to enhance lipid droplet formation in mast cells. [29] Our treatment of mast cell lipid droplets with 250 mmol of oleic acid for 18 to 20 hours induced the production of lipid droplets in mast cells (Fig. 16b, c), which is in accordance with the previous finding that the presence of extracellular oleic acid accelerates the formation of lipid droplets.[29] According to our data, primary in vitro-generated mature mast cells are capable of releasing PGD_2 in culture when activated via IgE receptor cross-linking. A notable increase in PGD_2 release after long term mast cell activation (Fig.7) was observed. Since AA-derived products are not stored but known to be synthesized de-novo, new enzyme molecules are likely to be synthesized in the mast cells during the activation since their production lasted up to 48 hours. On the other hand, these enzymes might be very stable with a long half-life. COX-2 is known to be degraded rapidly, whereas COX-1 and HPGDS are considered rather stable at least in megakaryocytes with “normal” intracellular levels of calcium.[20] Long term release of AA-derived products also requires large amounts of arachidonic acid. Indeed, mast cell lipid droplets contain much of arachidonic acid esterified in triglycerides, which might be released by ATGL and/or HSL and further used for PGD_2 production. Triglyceride -hydrolyzing lipase(s) able to respond to cell activation and to mobilize

arachidonic acid producing PGD_2 must be available in the droplet, if the lipid droplet actually is an immediate source for arachidonic acid.

We analyzed the gene expression of these putative candidate lipases, ATGL and HSL, as well as the ATGL co-activating factor CGI-58, in resting and activated mast cells. We also analyzed the mRNA expression levels of the prostaglandin D synthase HPGDS, which is the major PGD_2-forming enzyme in human mast cells[47]. According to our qPCR, Western blot and immunofluorescence data mast cells expressed HPGDS (Fig. 6f and 18). A scientific article supporting this observation was published while the writing of this thesis was still in progress.[48] In our experiments, the relative expression of HPGDS increased already in IgE treated mast cells compared to control treated cells and the expression was even greater in mast cells treated with αIgE also (Fig.18). Although the expression of HPGDS seems to be different in IgE-stimulated and unstimulated mast cells, this cannot be said for sure on the basis of one experiment and would require more detailed analysis. However our findings are supported by the previous finding that HPGDS can be up-regulated[5] and induced by KIT in immature mouse bone marrow derived mast cells.[28,49] An intracellular protein RasGRP4[2,5,8,49] that regulates the expression of HPGDS gene,[49,50] the presence on DAG which has been shown to modify the RasGRP4- mediated effects on HPGDS gene expression;[2,5] or MITF[50] that regulates the expression of Kit and specifically activates the cyclooxygenase pathway by binding to the HPGDS gene promoter region[50] might also enhance the generation of PGD_2 at least in mouse bone marrow mast cells.[2,50] It is not known if any other mast cell surface receptors, besides the IgE receptor, regulate the expression of HPGDS as well.

No significant changes were observed in the mRNA expression levels of ATGL, HSL, CGI-58, HPGDS or COX-1 during mast cell activation (Fig. 6a-d, f). The only significant changes in mRNA expression levels were observed for COX-2 (Fig. 6e). Indeed, the expression level of this inducible cyclooxygenase increased more than 40-fold one hour after activation and after that the peak sharply declined. This is in accordance with the previous knowledge about the phasic response of the PGD_2 release[11,13,28,50] and the role of COX-2 as an inducible enzyme. The sudden decrease in COX-2 amount after 2 hours might result from a negative regulation mediated by a PGD_2 metabolite, the 15-deoxy- Δ 812,149-PGJ2, which would then also reduce the amount of PGD_2 released.[8] Even though there were no changes in mRNA levels of ATGL and HSL and CGI-58 in activated mast cells, their enzymatic activities may have been changed[39]. The correlation between gene expression and protein levels depends on the cell type and experimental conditions, which may induce diverse changes in the regulation of mRNA and protein levels. Actually, the correlation

can be affected by changes in the degradation rate of mRNA, alternative splicing, protein stability and post translational modifications.[51] Therefore, protein levels cannot be deduced straightforward from the changes in mRNA levels or vice versa.

According to our immunofluorescence staining, both ATGL and HPGDS were recognized throughout the cytosolic area in the non-activated Ctrl cells, and hence the localization of ATGL in mast cells resembles that of the adipocytes[19]. Although HPGDS located in the circumference of mast cells, no clear localization of HPGDS was observed in the circumference of mast cell lipid droplets. This would have been expected based on previous findings about the localizations of synthetic enzymes for leukotrienes and PGs within the lipid droplets[1] or of COXs on the lipid droplet surface[23]. We do not know whether HPGDS even needs to be located on the lipid droplet surface, but visualization of HPGDS on the lipid droplet surface by confocal microscopic studies might provide an answer to this question. The use of confocal microscopy would be mandatory, since it is difficult to reveal any co-localization of HPGDS with lipid droplets by using normal fluorescence microscopy because the very strong HPGDS expression gives also a very strong signal when performing immunofluorescence staining. In order to establish the presence of HPGDS on lipid droplets, fractionation experiments to isolate pure lipid droplet fractions for testing the presence of HPGDS on lipid droplets by aid of Western blotting would also be needed. In the present work such lipid droplet isolation was not pursued, since it would have required large quantity of biological material and careful analysis using various organelle markers to exclude cross-contaminations.

In order to study the possibility that the triglyceride -hydrolyzing lipase(s) ATGL and HSL are able to respond to cell activation and to mobilize arachidonic acid for PGD_2 production in mast cells, siRNA transfection experiments were performed. Silencing the ATGL gene or HSL gene in human mast cells did not reveal any significant reduction of PGD_2 release, when analyzing the release of this lipid mediator during the early phase (30 to 120 min after activation) of mast cell activation (Fig. 8, 12). The HSL single silencing somewhat reduced the amount of PGD_2 produced in the early phase, but not significantly (Fig. 8).These observations are in contradiction with a latter study conducted at the Wihuri Research Institute, in which ATGL single silencing was observed to reduce the amount of eicosanoids produced.[52] As a consequence, we also included analysis of a shorter time point, just in case the reduction in PGD_2 levels had occurred faster and already happened during the acute phase of activation, i.e. during the time period spanning from the moment of activation until 15 minutes after the activation. We studied the effect of HSL and ATGL single and

double silencing during the acute PGD_2 release, and surprisingly, could observe a clear reduction in PGD_2 levels compared to control-silenced mast cells (Fig 13). HSL knockdown showed a clear reduction (60% of control cells after 5 minutes and 70% after 15 minutes) in PGD_2 release in acute phase compared to control cells, and in ATGL single-silenced mast cells the reduction was even more pronounced (27% after 5 minutes and 48% after 15 minutes) probably because ATGL catalyzes the rate limiting step of triglyceride hydrolysis. By far the strongest reduction in the acute PGD_2 release was seen with the ATGL+HSL –double knockdown, likely because both enzymes are able to start hydrolyzing triglycerides. The release was 17% of that of the controls after 5 minutes and 37% after 15 minutes. ATGL and HSL are probable candidates for mobilizing arachidonic acid from triglycerides in mast cell lipid droplets for the use in biosynthesis of PGD_2 since knockdown of these two enzymes cuts down the release of PGD_2. Another possible fate of the arachidonic acid released from triglycerides is the lipid droplet monolayer, where arachidonic acid might get re-esterified into phospholipids, and then released again by the action of phospholipases.[53]

The challenges for the generation of primary mast cells from circulating progenitor cells derived from adult peripheral blood include donor variability and accessibility, the possibility of contamination with other cell types and microbiological organisms, compromised mast cell survival and growth in culture, and finally, also variation in the transfection efficiency. The ATGL and HSL knockdowns were successful as judged by reduced transcript levels in qPCR. However, these knockdowns were not strong enough to cut down the early phase of PGD_2 production. Despite this failure, we propose that HSL and ATGL might be the ones mainly affecting the amount of PGD_2 released from activated mast cells in the acute and early phase of PGD_2 release, but that also some other factors likely play a role. The ramifications of this study include the possibility that arachidonic acid release from triglycerides for the formation of eicosanoids could take an indirect or a direct route to supply precursors for cellular eicosanoid biosynthesis (Fig. 19; left and right, respectively).

The proposed indirect pathway in mast cells could involve release of PGD_2 through a more regulated pathway (Fig. 19, on the left-hand side). In this indirect pathway, arachidonic acid is released from triglycerides present in the lipid droplets by ATGL and/or HSL, then activated by ACSL3 or ACSL4 and included into lipid droplet membrane phospholipids (PLs). cPLA could release this incorporated arachidonic acid which could be then converted to PGH for lipid mediator generation by the COX-1 enzyme whose expression stays up until the onset of mast cell activation. Phospholipase A_2- family consist of 15 different isoenzymes, which all enzymatically hydrolyze sn-

2 -ester bonds of glycerophospholipids releasing fatty acids (AA) and lysophospholipids (lysophosphatidylcholine, LPC). cPLA2 is a multi-compartment enzyme whose function as a major arachidonic acid releasing enzyme is well established.[35,37,39] It is not so long ago that arachidonic acid released from phospholipids of various membranous structures by cPLA was held as the only substrate for eicosanoid biosynthesis.[33] The experiments carried out at the Wihuri Research Institute, including those presented here, have established that, in addition to phospholipids, the triglycerides present in mast cell lipid droplet core also are an important source of eicosanoids, and that also ATGL and HSL, not just cPLA, can release arachidonic acid for eicosanoid production.

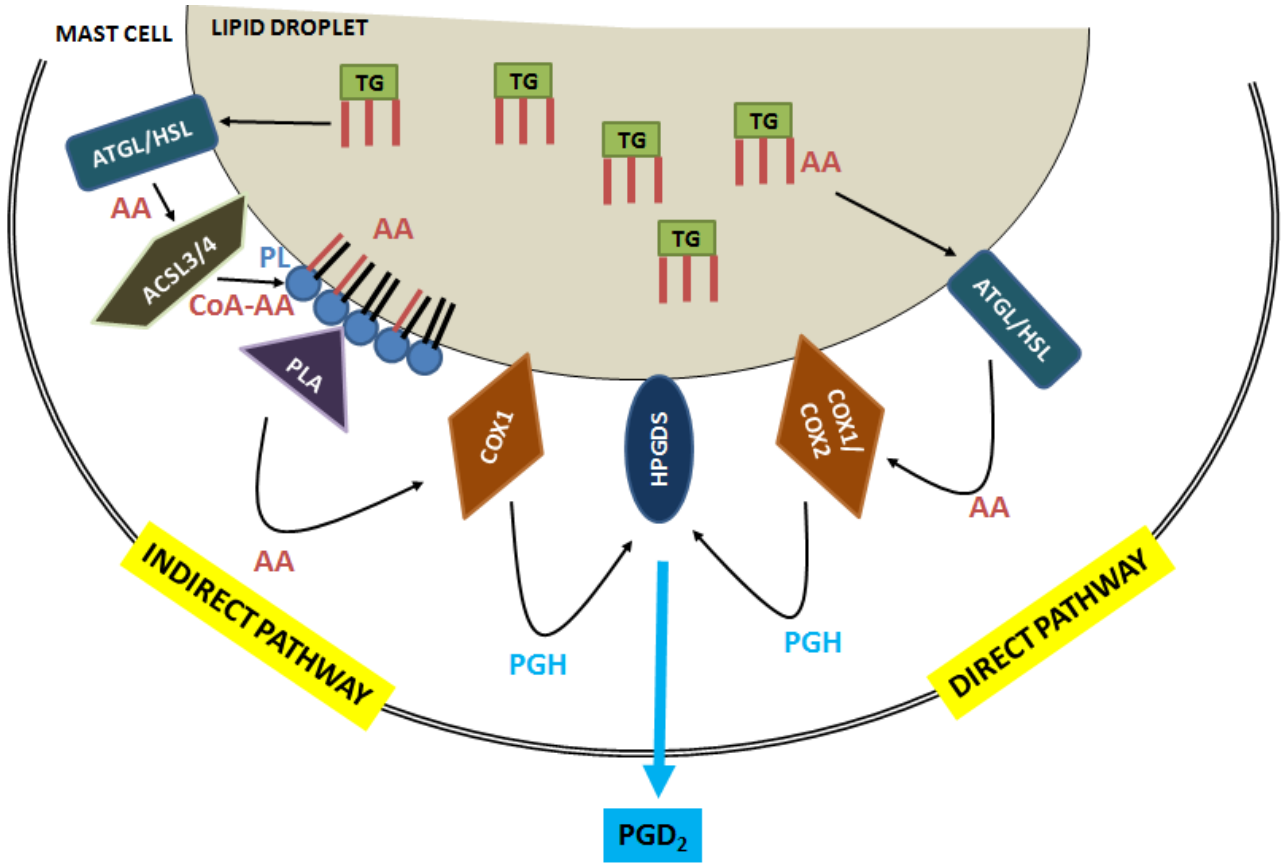


Figure 19: Acute and short term release of PGD_2 from TG-derived AA stored in human MC LDs. Human mast cells could release PGD_2 from triglyceride-derived arachidonic acid stored in human mast cell lipid droplets via direct or indirect pathways. In the direct pathway (on the right), arachidonic acid is released by ATGL or HSL from lipid droplet triglycerides and this free arachidonic acid is used by COX-1 or COX-2 depending on the status of the cell for the generation of prostaglandins. In the indirect pathway (on the left-hand side), arachidonic acid is liberated from lipid droplet triglycerides by ATGL or HSL and then further re-esterified into phospholipids from where arachidonic acid can then finally be released by cPLA2 for the generation of eicosanoids. Abbreviations: ATGL = adipose triglyceride lipase, HSL = hormone-sensitive lipase, ACSL = long chain acyl-CoA synthetase, cPLA2 = cytosolic phospholipase A2, COX = cyclooxygenase 1 and 2, HPGDS = hematopoietic prostaglandin D synthase, AA = arachidonic acid, PGH = prostaglandin H, PGD_2 = prostaglandin D2, TG = triglyceride, AA = arachidonic acid, MC = mast cell, LD = lipid droplet. Figure modified from Dichlberger et al.[52]

Under conditions of immunological mast cell stimulation (Fig. 4), the arachidonic acid present in the lipid droplets could be released from triglycerides via a direct pathway (Fig. 19; on the right-hand side). In the proposed direct pathway arachidonic acid is released from the triglycerides present in the lipid droplets of mast cells by CGI-58-activated ATGL and HSL and the free arachidonic acid is then converted to PGH by either COX-1 under basal conditions and/or COX-2 under activated conditions. This presumption is supported by the fact that the basal and immediate PGD_2 release is mediated by COX-1 which is also constitutively expressed in mast cells (Fig. 6d).[24] Incubation of the constitutively expressed COX-1 with aspirin has been shown to irreversibly inhibit the release of PGD_2 during the first 30 minutes after mast cell activation.[52] This enzyme is believed to be responsible for the immediate phase of the eicosanoid release in unstimulated mast cells, while COX-2 is believed to be responsible for the delayed and stimulated phase of PGD_2 release.[13,24,52] The second, delayed but pronounced phase of PGD_2 generation was visibly affected by ATGL and HSL knockdowns in a reducing manner. This phase of PGD_2 generation is likely elicited by KIT and certain interleukins, is regulated by exogenous factors controlling the expression levels of COXs and by the availability of cytokines,[11] requires mast cell sensitization with IgE and is mediated by and dependent on COX-2[13,24] (Fig. 19, on the right-hand side), the gene expression of which was highly up-regulated as late as after 1 hour of activation (Fig. 6e). However, the effect of KIT on COX-2 induction and expression is somewhat controversial.[13,28,50] Moreover, in some studies the KIT-stimulated increase in the expression of cPLA2, COX-1 and HPGDS have been shown to lead to a selective increase in IgE-dependent production of PGD_2 from endogenous arachidonic acid.[28]

Regardless of the pathway of releasing arachidonic acid -either directly from triglycerides for the generation of eicosanoids or indirectly via the phospholipids, HPGDS could convert the PGH into PGD_2 which in turn would be released from the lipid droplet into the mast cell cytosol. It has been reported that mice having a disrupted HPGDS gene are unable to produce PGD_2 both during the acute and delayed phases.[50] Since both phases of PGD_2 production depend on HPGDS, the enzyme likely plays its role(s) at the intersection of the direct and indirect pathways of arachidonic acid liberation and PGD_2 production from both the triglyceride core and the phospholipid monolayer of the mast cell lipid droplet. In accordance with this presumption is the finding that the amount of PGD_2 produced is determined mainly by the amount of HPGDS in the cell while KIT also in some way regulates the amount and expression of HPGDS.[49] IL-3 also has minor effects on HPGDS levels.[49]

6 Closing words

Prostaglandin levels are generally low in non-inflamed tissues, but during inflammation, the levels and the molecular profiles of the prostaglandins produced changes markedly.[21] Indeed, prostaglandins have been found to be important both in the acute phase and the resolution phase of inflammation.[11] Acute inflammation begins with the production of soluble mediators by the cells residing in the injured or infected tissue.[14] The cells include mast cells that contribute to the inflammation by releasing both preformed mediators such as histamine and newly formed mediators, such as the eicosanoids.[13,14] Different eicosanoids derived from arachidonic acid can either alleviate or worsen the inflammatory responses and influence the magnitude, nature and duration of the immune responses. Even the same eicosanoid produced by either COX-1 or COX-2 may in different situations promote or help resolve the inflammation.[11,21]

PGD_2 production by mast cells initiates IgE-mediated allergic responses in the body[21] by acting as a local mediator in an autocrine and paracrine fashion. The presence of an allergic antigen can induce the production PGD_2 in sensitized individuals; for example, in patients suffering from allergic asthma, PGD_2 released by pulmonary mast cells can be detected from bronchoalveolar lavage fluid during an asthma attack.[21,49] PGD_2 causes a contraction of the bronchial airways, and because of the strong mechanistic link of such contraction to the pathogenesis of this disease, several drugs that seek to reduce PGD_2 levels in asthma have already been tested in clinical trials.

PGD_2 may also play a role in the evolution of atherosclerosis.[21] In the inflamed arterial intima of an atherosclerotic arterial segment, the amount of PGD_2 increases because inflammatory cells producing HPGDS show positive chemotaxis and permeate the vasculature.[21] The expression and presence of ATGL in human mast cells and its importance for the generation of PGD_2 from mast cell lipid droplets has by now been published from Wihuri Research Institute, [52] while the presence of CGI-58 is so far an unpublished observation. In addition to ATGL and HSL, also other enzymes likely are important for the triglyceride lipolysis, fat physiology and eicosanoid biosynthesis – especially for the generation of PGD_2 in mast cells. Based on their newly discovered roles in inflammation, ATGL and HSL might even be potential targets for new drugs. However, since they are widely distributed in the human body, notably having physiological roles in the adipose tissue, a tissue-targeted or a cell-targeted approach might be necessary to selectively influence their functions in pathophysiologically relevant pathways. Even without any direct clinical benefits, basic research on these triglyceride lipases helps figuring out the mechanism and pathways of eicosanoid biosynthesis in mast cells and other inflammatory cells, and thereby widens our understanding

about the pathways leading to the PGD_2 synthesis and the progressions of inflammatory diseases, such as asthma and atherosclerosis.

7 Acknowledgements

The Wihuri Research Institute maintained by the Jenny and Antti Wihuri Foundation and supervisors professor Petri Kovanen and PhD Andrea Dichlberger.

8 CV

Presentations

2012 Dichlberger A, Schlager S, **Tentke A**, Schneider WJ, Kovanen PT. Lipid bodies in human mast cells: a distinct source of arachidonic acid for eicosanoid biosynthesis. 5th MacNet Meeting, August 2012, Säröhus, Sweden

9 References

1. Greineisen WE, Shimoda LM, Maaetoft-Udsen K, Turner H. Insulin-containing lipogenic stimuli suppress mast cell degranulation potential and up-regulate lipid body biogenesis and eicosanoid secretion in a PPARγ-independent manner. *J Leukoc Biol*. 2012;92(3):653-665.

2. Okayama Y, Kawakami T. Development, migration, and survival of mast cells. *Immunol Res*. 2006;34(2):97-115.

3. Marshall JS. Mast-cell responses to pathogens. *Nature Reviews Immunology*. 2004;4(10):787-799.

4. Kirshenbaum AS, Akin C, Wu Y, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res*. 2003;27(8):677-682.

5. Kanaoka Y, Ago H, Inagaki E, et al. Cloning and crystal structure of hematopoietic prostaglandin D synthase. *Cell*. 1997;90(6):1085-1095.

6. Valent P, Spanblochl E, Sperr WR, et al. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood*. 1992;80(9):2237-2245.

7. Yanagida M, Fukamachi H, Ohgami K, et al. Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood*. 1995;86(10):3705-3714.

8. Yang Y, Li L, Wong GW, et al. RasGRP4, a new mast cell-restricted ras guanine nucleotide-releasing protein with calcium-and diacylglycerol-binding motifs identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function. *J Biol Chem*. 2002;277(28):25756-25774.

9. Lennartsson J, Rönnstrand L. Stem cell factor receptor/c-kit: From basic science to clinical implications. *Physiol Rev*. 2012;92(4):1619-1649.

10. Oksaharju A. Effects of proatherogenic and probiotic bacteria on mast cells and inflammation. Helsinki University Print. 2012 (dissertation).

11. Boyce JA. Mast cells and eicosanoid mediators: A system of reciprocal paracrine and autocrine regulation. *Immunol Rev*. 2007;217(1):168-185.

12. Dichlberger A, Kovanen PT, Schneider WJ. Mast cells: From lipid droplets to lipid mediators. *Clin Sci*. 2013;125(3):121-130.

13. Diaz BL, Fujishima H, Kanaoka Y, Urade Y, Arm JP. Regulation of prostaglandin endoperoxide synthase-2 and IL-6 expression in mouse bone marrow-derived mast cells by exogenous but not endogenous prostanoids. *The Journal of Immunology*. 2002;168(3):1397-1404.

14. Stables MJ, Gilroy DW. Old and new generation lipid mediators in acute inflammation and resolution. *Prog Lipid Res*. 2011;50(1):35-51.

15. Laidlaw TM, Steinke JW, Tiñana AM, et al. Characterization of a novel human mast cell line that responds to stem cell factor and expresses functional FcεRI. *J Allergy Clin Immunol*. 2011;127(3):815-822.e5. doi: http://dx.doi.org/10.1016/j.jaci.2010.12.1101.

16. Lappalainen J, Lindstedt K, Kovanen P. A protocol for generating high numbers of mature and functional human mast cells from peripheral blood. *Clinical & Experimental Allergy*. 2007;37(9):1404-1414.

17. Calder PC. Polyunsaturated fatty acids and inflammation. *Prostaglandins, leukotrienes and essential fatty acids*. 2006;75(3):197-202.

18. Rink C, Khanna S. Significance of brain tissue oxygenation and the arachidonic acid cascade in stroke. *Antioxidants & Redox Signaling*. 2011;14(10):1889-1903.

19. Brasaemle DL. Thematic review series: Adipocyte biology. the perilipin family of structural lipid droplet proteins: Stabilization of lipid droplets and control of lipolysis. *J Lipid Res*. 2007;48(12):2547-2559.

20. Yazaki M, Kashiwagi K, Aritake K, Urade Y, Fujimori K. Rapid degradation of cyclooxygenase-1 and hematopoietic prostaglandin D synthase through ubiquitin–proteasome system in response to intracellular calcium level. *Mol Biol Cell*. 2012;23(1):12-21.

21. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*. 2011;31(5):986-1000.

22. Rajakariar R, Hilliard M, Lawrence T, et al. Hematopoietic prostaglandin D2 synthase controls the onset and resolution of acute inflammation through PGD2 and 15-deoxyΔ12–14 PGJ2. *Proceedings of the National Academy of Sciences*. 2007;104(52):20979-20984.

23. Dvorak A, Dvorak H, Peters S, et al. Lipid bodies: Cytoplasmic organelles important to arachidonate metabolism in macrophages and mast cells. *The Journal of immunology*. 1983;131(6):2965-2976.

24. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: Structural, cellular, and molecular biology. *Annu Rev Biochem*. 2000;69(1):145-182.

25. Urade Y, Hayaishi O. Biochemical, structural, genetic, physiological, and pathophysiological features of lipocalin-type prostaglandin D synthase. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*. 2000;1482(1):259-271.

26. Satoshi K, Young-Ho L, Yuya M, Harumi F, Yuji G, Takashi I. Systematic interaction analysis of human lipocalin-type prostaglandin D synthase with small lipophilic ligands. *Biochem J*. 2012;446(2):279-289.

27. Hyo S, Kawata R, Kadoyama K, et al. Expression of prostaglandin D2 synthase in activated eosinophils in nasal polyps. *Archives of Otolaryngology—Head & Neck Surgery*. 2007;133(7):693-700.

28. Murakami M, Matsumoto R, Urade Y, Austen KF, Arm JP. C-kit ligand mediates increased expression of cytosolic phospholipase A, prostaglandin endoperoxide synthase-1, and hematopoietic prostaglandin D synthase and increased IgE-dependent prostaglandin D generation in immature mouse mast cells. *J Biol Chem*. 1995;270(7):3239-3246.

29. Dichlberger A, Schlager S, Lappalainen J, et al. Lipid body formation during maturation of human mast cells. *J Lipid Res*. 2011;52(12):2198-2208.

30. Wang H, Hu L, Dalen K, et al. Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins. *J Biol Chem*. 2009;284(46):32116-32125.

31. Murphy DJ. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog Lipid Res*. 2001;40(5):325-438.

32. Zweytick D, Athenstaedt K, Daum G. Intracellular lipid particles of eukaryotic cells. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*. 2000;1469(2):101-120.

33. Bozza PT, Bakker-Abreu I, Navarro-Xavier RA, Bandeira-Melo C. Lipid body function in eicosanoid synthesis: An update. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2011;85(5):205-213.

34. Wang H, Bell M, Sreenevasan U, et al. Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *J Biol Chem*. 2011;286(18):15707-15715.

35. Moreira LS, Piva B, Gentile LB, et al. Cytosolic phospholipase A2-driven PGE2 synthesis within unsaturated fatty acids-induced lipid bodies of epithelial cells. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2009;1791(3):156-165. doi: http://dx.doi.org/10.1016/j.bbalip.2009.01.003.

36. Kovanen PT. Mast cells: Multipotent local effector cells in atherothrombosis. *Immunol Rev*. 2007;217(1):105-122.

37. Pérez-Chacón G, Astudillo AM, Balgoma D, Balboa MA, Balsinde J. Control of free arachidonic acid levels by phospholipases A< sub> 2 and lysophospholipid acyltransferases. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*. 2009;1791(12):1103-1113.

38. Bazan NG. Arachidonic acid in the modulation of excitable membrane function and at the onset of brain damagea. *Ann N Y Acad Sci*. 1989;559(1):1-16.

39. Lass A, Zimmermann R, Oberer M, Zechner R. Lipolysis–a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. *Prog Lipid Res*. 2011;50(1):14-27.

40. Lampidonis AD, Rogdakis E, Voutsinas GE, Stravopodis DJ. The resurgence of hormone-sensitive lipase (HSL) in mammalian lipolysis. *Gene*. 2011;477(1):1-11.

41. Kraemer FB, Shen W. Hormone-sensitive lipase control of intracellular tri-(di-) acylglycerol and cholesteryl ester hydrolysis. *J Lipid Res*. 2002;43(10):1585-1594.

42. Granneman JG, Moore HH, Mottillo EP, Zhu Z. Functional interactions between mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *J Biol Chem*. 2009;284(5):3049-3057.

43. Melo RC, D'Avila H, Wan H, Bozza PT, Dvorak AM, Weller PF. Lipid bodies in inflammatory cells structure, function, and current imaging techniques. *Journal of Histochemistry & Cytochemistry*. 2011;59(5):540-556.

44. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nature protocols*. 2008;3(6):1101-1108.

45. Dvorak AM, Morgan E, Schleimer RP, Ryeom SW, Lichtenstein LM, Weller PF. Ultrastructural immunogold localization of prostaglandin endoperoxide synthase (cyclooxygenase) to non-membrane-bound cytoplasmic lipid bodies in human lung mast cells, alveolar macrophages, type II pneumocytes, and neutrophils. *J Histochem Cytochem*. 1992;40(6):759-769.

46. Triggiani M, Oriente A, Seeds MC, Bass DA, Marone G, Chilton FH. Migration of human inflammatory cells into the lung results in the remodeling of arachidonic acid into a triglyceride pool. *J Exp Med*. 1995;182(5):1181-1190.

47. Urade Y, Ujihara M, Horiguchi Y, et al. Mast cells contain spleen-type prostaglandin D synthetase. *J Biol Chem*. 1990;265(1):371-375.

48. Trujillo JI, Kiefer JR, Huang W, et al. Investigation of the binding pocket of human hematopoietic prostaglandin (PG) D2 synthase (hH-PGDS): A tale of two waters. *Bioorg Med Chem Lett*. 2012;22(11):3795-3799.

49. Li L, Yang Y, Stevens RL. RasGRP4 regulates the expression of prostaglandin D2in human and rat mast cell lines. *J Biol Chem*. 2003;278(7):4725-4729.

50. Morii E, Oboki K. MITF is necessary for generation of prostaglandin D2 in mouse mast cells. *J Biol Chem*. 2004;279(47):48923-48929.

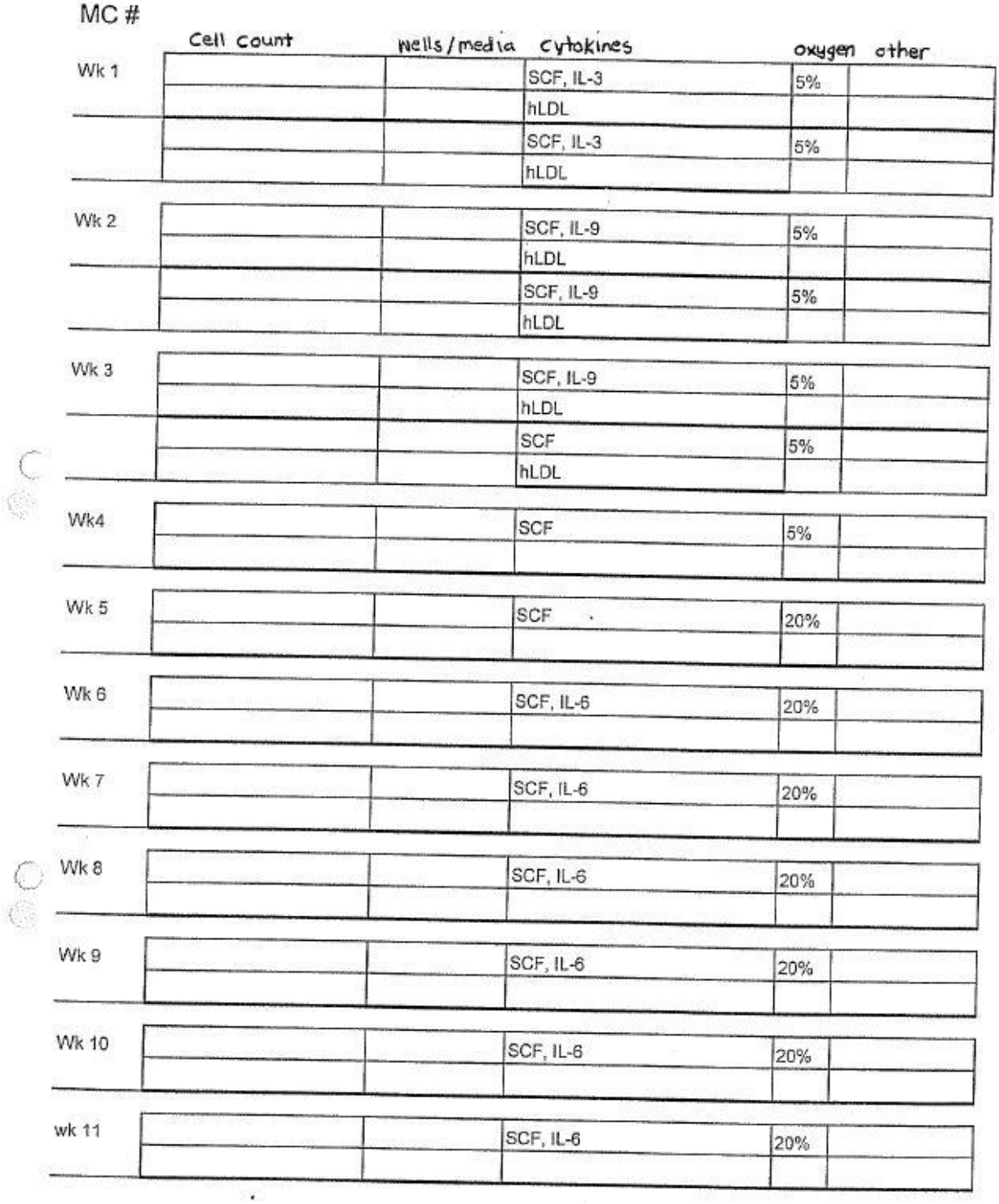
51. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics*. 2012;13(4):227-232.

52. Dichlberger A, Schlager S, Maaninka K, Schneider WJ, Kovanen PT. Adipose triglyceride lipase regulates eicosanoid production in activated human mast cells. *J Lipid Res*. 2014. doi: jlr.M048553 [pii].

53. Soupene E, Kuypers FA. Mammalian long-chain acyl-CoA synthetases. *Exp Biol Med*. 2008;233(5):507-521.

10 Attachments

Attachment 1: Weekly plan for mast cell maintaining. [16]



MC #

	Cell count		Wells / media	Cytokines	oxygen	other
Wk 1				SCF, IL-3	5%	
				hLDL		
				SCF, IL-3	5%	
				hLDL		
Wk 2				SCF, IL-9	5%	
				hLDL		
				SCF, IL-9	5%	
				hLDL		
Wk 3				SCF, IL-9	5%	
				hLDL		
				SCF	5%	
				hLDL		
Wk4				SCF	5%	
Wk 5				SCF	20%	
Wk 6				SCF, IL-6	20%	
Wk 7				SCF, IL-6	20%	
Wk 8				SCF, IL-6	20%	
Wk 9				SCF, IL-6	20%	
Wk 10				SCF, IL-6	20%	
wk 11				SCF, IL-6	20%	